CLONAL PROPAGATION OF *Tylophora indica*- AN IMPORTANT MEDICINAL PLANT THROUGH TISSUE CULTURE

A

Desertation Report

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CERTIFICATE

This is to certify that the thesis entitled **"Clonal Propagation of *Tylophora indica*- An important Medicinal Plant Through Tissue Culture**" submitted by Harleen Kaur Nadha in partial fulfillment of the requirements for the award of degree of Masters of science in Biotechnology to Thapar Institute of Engineering and Technology, Patiala, is a record of student's own work carried out by her under our supervision and guidance. The report has not been submitted for the award of any other degree or certificate in this or any other University or Institute.

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ABSTRACT

The present investigation was carried out on an important medicinal plant *Tylophora indica* belonging to the family Asclepiadaceae. The different vegetative parts i.e. stem, leaf and shoot apices & nodal explants were excised from an elite field grown mature plant and thereafter planted on variously supplemented Murashige and Skoog's medium for callus induction, organagenesis and multiple shoot proliferation.

Tylophora exhibited high degree of multiple shoot proliferation from nodal segments taken from *in vivo* plants. Prolific multiple shoot regeneration from nodal explants was observed on MS medium + BAP (1-4ppm), MS + BAP (1-4ppm) + CM (15%). Best results were however obtained on 2ppm BAP, where 45-50 shoots were obtained after 7-8 weeks of culturing. With increase in the concentration of BAP number of shoots formed had waned. Shoots thus obtained were excised & planted on MS medium supplemented with IBA & IAA for induction of roots. Best results were however obtained on MS supplemented with IBA (4ppm).

Callus formation occurred from nodal segments, leaf and stem explants (internodal segments) when planted on different combinations of auxins & cytokinins. Murashiege & Skoog's agar gelled medium supplemented with NAA (4ppm) + K (1ppm) turned out to be optimal for initiation & sustained growth of calli from all the three parts. Callus was also obtained on 2,4-D (4ppm) + Kinetin (1ppm) and BAP (1-6ppm) but time taken for its formation was quite long. The calli thus formed were green, solid & fast growing. The calli induced from different parts on the same medium were more or less identical in morphology. The calli obtained were heterogenous being composed of parenchymatous ovoid, oblong, semicircular cells or those with aberrant shapes.

The spectrum of induced differentiation from calli was wide and involved xylogenesis, rhizogenesis and caulogenesis. The trachieds occurred either singly or were grouped together as nodules. They had sclariform thickenings on their

walls. Root formation occurred after 3 weeks when callus was planted on MS medium supplemented with different concentrations of IBA (0.5-4ppm). Roots obtained were long, white, and branched. Shoot formation from callus was obtained on MS + BAP (0.5-4ppm). Nearly 5-8 shoots were obtained after 4 weeks of culturing. Shoots thus obtained were rooted on a separate root inducing media consisting of different concentrations of IBA but best results were obtained on MS + IBA (4ppm). The plantlets with elongated root and shoot system were then subjected to hardening and attempts were made to establish these plantlets into the soil.

CONTENTS

1. INTRODUCTION

Human beings have been utilizing plants for basic preventive and curative health care since time immemorial. Recent estimates suggest that over 9,000 plant species have known medicinal applications in various cultures and countries and this is without having conducted comprehensive research amongst several indigenous and other communities (Farnsworth and Soejarto 1991).

The widespread use of herbal remedies and health care preparations, as described in ancient texts including the Vedas, holy Koran and the Bible are obtained from commonly used traditional herbs and medicinal plants. In India, approximately 1700 plant species are used in Ayurveda, 500 for Siddha, 400 for Unani, 300 for Amchi systems of medicine with substantial overlaps of common plants among these systems.

The trend of using natural products is increasing steadily. The use of traditional medicines and medicinal plants in most developing countries as a normative basis for maintenance of good health has been widely observed. Further an increasing reliance on the use of medicinal plants in the industrialized societies has been related to the development of several drugs and chemotherapeutics from plant species as well as from traditionally used rural herbal preparations. Herbal remedies have attained much more popularity in the treatment of minor ailments, due to increasing awareness of personal health maintenance through natural products. Indeed, the market and public demand has been so great that there is a great extinction risk to many medicinal plants and obviously the loss of genetic diversity.

In view of the growing world population, increasing anthropogenic activities, rapidly eroding natural ecosystem etc the natural habitat for a

great number of herbs and trees are dwindling. Many of them are facing extinction. To cope up with alarming situation the recent exciting developments in biotechnology have come as a boon. One of them is the use of plant tissue culture technique. Most of the plant raised through seeds are highly heterozygous and show great variations in growth, habit and yield and may have to be discarded because of poor quality of products for their commercial release. Likewise, majority of the plants are not amenable to vegetative propagation through cutting and grafting, thus limiting multiplication of desired cultivars. Moreover many plants propagated by vegetative means contain systemic bacteria, fungi and viruses which may affect the quality and appearance of selected items.

In recent years, tissue culture has emerged as a promising technique to obtain genetically pure elite populations under *in vitro* conditions rather than have indifferent populations. Tissue culture has now become a well established technique for culturing and studying the physiological behavior of isolated plant organs, tissues, cells, protoplasts and even cell organelles under precisely controlled physical and chemical conditions. *In vitro* propagation also called micropropagation is in fact the miniature version of conventional propagation which is carried out under aseptic conditions.

Most of the medicinal plants either don’t produce seeds or seeds are too small & don’t germinate in soils. Thus mass multiplication of disease free planting material is a general problem. In this regard the micropropagation holds significant promise for true to type, rapid & mass multiplication under disease free conditions. Besides, the callus derived plants exhibit huge genetic variation that could be exploited for developing superior clones/varieties particularly in vegetatively propagated plant species.

Global overview of Medicinal plants

Plants, since time immemorial, have been used globally across the varied cultures throughout the known civilizations as a valuable and safe natural source of medicine and agents of therapeutic, industrial and environmental utilities. Medicinal plants are used at the household level by women taking care of their families, at the village level by medicine men or tribal shamans and by the practitioners of classical traditional systems of medicine such as Ayurveda, Chinese medicine or the Japanese Kampo system. According to the World Health Organization, over 80% of the world's population or 4.3 billion people rely upon such traditional plant-based systems of medicine to provide them with primary health care.

Allopathic medicine too owes a tremendous debt to medicinal plants: one in four prescriptions filled in a country like the United States is either a synthesized form of or derived from plant materials. Even from the earliest trade data available, it is clear that the global market for medicinal plants has always been very large. It is only during the last decade that the real significance of the medicinal plants sector has begun to be realized.

Current trends all over the world have shown that for one reason or the other, people are not only willing to try natural medicine especially those of plant origin but also activity seeking nonconventional remedies. As a result there is a global resurgence in the trade of herbal medicines. International market of medicinal plants is reported to be over 62 billions US dollars per year during 2000-2001, which is growing at the rate of 7% annually. The botanical retail market, inclusive of herbs and medicinal plants, in USA, is estimated at approximately US$1.6 billion annually. It is estimated that Europe annually imports about 400,000 tonnes of medicinal plant material with an average market value of US$1 billion from Africa and Asia. A growing awareness of this new contributor to the foreign

exchange reserves of several national treasuries is beginning to emerge. To satisfy the growing market demands, surveys worldwide are being conducted by the pharmaceutical industries and research organization to unearth new plant sources as herbal remedies, medicines and biomolecules.

Status of Medicinal plants in India

Medicinal plants as a group comprise approximately 8000 species and account for about 50% of all the higher flowering plant species of India. Millions of rural mass use medicinal plants. In recent years the growing demand for herbal products has led to a quantum jump in volume of plant material traded within and outside the country. An estimate of the EXIM Bank projects international market of medicinal plants related trade over US $ 60 billion per year that is growing at a rate of 7% per year.

India's surface land has been grouped into ten distinct zones and these are further divided into 25 biotic provinces and 426 biomes. The forest areas of these bio-geographic zones/provinces are classified into 16 major forest types and more than 200 subtypes. A macro analysis of the distribution of medicinal plants shows around 70% of India's medicinal plants are found in tropical and subtropical forests and less than 30% are found in the temperate and high altitude forests. The medicinal plants species belong to a wide range of habits/life form viz. trees, herbs, shrubs, lianas, and woody climbers. Habit wise analysis carried out so far indicates that nearly one third of these botanical entities are trees and around the same proportion consists of shrubs and woody climbers. The remaining one third are herbs and twiners. Very small proportions of the medicinal plants are lichens, ferns, algae etc; the majority of the medicinal plants are higher plants.

Though India has rich biodiversity and one among the twelve mega diversity centres, the growing demand is putting a heavy strain on the existing resources causing a number of species to be either threatened or endangered category. About 90% of medicinal plants used by industries are collected from the wild. While over 800 species are used in production by industry, less than 20 species of plants are under commercial cultivation. Over 70% of the plant collections involve destructive harvesting because of the use of parts like roots, bark, wood, stem and the whole plant in case of herbs. This poses a definite threat to the genetic stocks and to the diversity of medicinal plants, if biodiversity is not sustainably used. Recently some rapid assessment of the threat status of medicinal plants using IUCN designed CAMP methodology revealed that about 112 species in southern India, 74 species in Northern and Central India and 42 species in the high altitude of Himalayas are threatened in the wild.

The need of the hour, then, is to replan India's participation in the expanding global market, in light of the interest of all the stakeholders who are affected and who play a role in this sector. There is a need to collect all the available information regarding medicinal plants development in the country in order to obtain a comprehensive overview which will provide the necessary insight for coordinated and effective action. Such an overview could form the basis of a renewed development of India's medicinal plants sector, and a strategic exploitation of her comparative advantage in the global market on a sustainable and equitable basis.

Important medicinal plants in India

The Indian system of medicine particularly Ayurveda, Siddha, Unani and Homeopathy largely use plant-based materials, minerals, metals,marine and products of animal origin. The domestic market of Indian system of medicine and Homeopathy is of order of Rs 4000 crores

which is expanding day by day. The Ayurveda drug market alone is of the order of Rs 3500 crores, besides this there are demands from food supplements and cosmetics. However, the sector is not well organized and needs special attention. Hence, the National Medicinal Plants Board was set up under the ministry of Heath and Family welfare, Govt. of India during 2000 and the board has initially identified 31 species of importance. In a report to the Scientific Advisory Committee to the cabinet (SAC-C), Govt. of India, Technology Information, Forecasting and Assessment Council (TIFAC) has mentioned 45 medicinal plant species and specifically recommended 7 plants for immediate attention during 2001-2005. They are as follows-

1. *Aloe vera* (Ghrita Kumari) 2. *Bacopa monnieri* (Brahmi) 3. *Centella asiatica* (Mandukparni, Gotukola), 4. *Rawolfia serpentine* (Sarpagandha), 5. *Catharanthus roseus* (Periwinkle) 6. *Taxus bacata* 7. *Artemisia annua*

Micropropagation

Most of the plants raised through seeds show tremendous genetic variations and the grower has to select from wide population the plants having the best characteristics. However, it is a very cumbersome & time consuming activity. Likewise majority of the plants are not amenable to vegetative propagation by culturing or grafting. *In vitro* culture technique also called micropropagation has become an important tool to obtain genetically pure elites rather than having indifferent populations.

Advantages of micropropagation

- Micropropagation can be used as an alternative to conventional methods of vegetative propagation with the objective of enhancing the rate of multiplication.

- ❖ Through *in vitro* clonal propagation, large number of plants can be raised from a small even microscopic piece of plant tissue within a short span of time.
- ❖ Micropropagation provides reliable and economical method of maintaining pathogen free plants in a state that can allow rapid multiplication and also allows international exchange of germplasm.
- ❖ Plant multiplication can continue throughout the year irrespective of season.
- ❖ Stocks of germplasm can be maintained for many years.

Techniques of Micropropagation

Three basic methods are used to propagate plants *in vitro.*

1) **Enhanced axillary shoot proliferation**

- Micropropagation through apical and axillary shoot proliferation is the most common method for commercial production.
- Cells of the meristems are uniformly diploid and are least susceptible to genetic changes. Hence most reliable technique for mass propagation since it ensures genetic stability of clones.

2) ***Denovo* formation of adventitious shoots**.

New adventitious shoots can develop either

a) Directly from the explants like root, stem, petiole, leaf lamina, flower parts etc or

b) Indirectly from callus cultures obtained from these explants. Plants obtained through calli may not be true elites because of high incidence of polyploidy & aneuploidy associated with callus cells & plants obtained from it.

3) **Somatic or nonzygotic embryogenesis**.

- Involves the formation of bipolar embryos which can develop into fully functional plants under appropriate conditions.

Stages in Micropropagation

Micropropagation involves 4 definite stages. These are as follow:-

Stage- I

Initiation and establishment of aseptic cultures:-

This involves explant isolation, surface sterilization and establishment on an appropriate culture medium. Cultures are initiated from explants of several organs but shoot apices and axillary buds are most often used for commercial micropropagation.

Stage- II

Shoot multiplication using a defined culture medium

It can be achieved through any one of the following four methods:-

- Multiplication through calli obtained from different organs and tissues and their subsequent subculturing leading to organogenesis.
- Multiplication through direct induction of shoots on the explants.
- Multiplication through growth and proliferation of existing apical shoots & adventitious buds.
- Somatic or nonzygotic embryogenesis directly on the explants or in callus cultures.

Stage- III

Rooting of regenerated shoots in *in vitro* conditions:-

This stage is characterized by preparation of stage II shoots or shoot clusters for successful transfer to the soil. The process may involve:-

- Elongation of shoots prior to rooting.
- Root formation:- Adventitious root formation involves 3 stages
  - Induction of rooting- it is the first stage in the process of rooting. In this process the fate of cell(s) is changed to root formation and results in the formation of root primordium.

- Emergences of roots- root primordium grows and emerges out from the epidermis.
- Elongation of roots- further elongation of root primordium results in root formation.

Stage-IV

Transfer of plantlets to natural environment (acclimatization):-

- The climatic adaptation of a plant when moved to a new environment is known as acclimatization.
- Plants which are produced under cultural conditions (high humidity, low light, constant temperature) when transferred to field conditions are required to be acclimatized.
- The ultimate success of any micropropagation protocol depends upon the ability to transfer and reestablish plants from *in vitro* to green house conditions. Micropropagated plants are difficult to transplant for two primary reasons:-
  - ❖ A heterotrophic mode of nutrition.
  - ❖ Poor control of water loss.

  To overcome these limitations, plantlets should be transplanted into a well drained, sterile growing medium and maintained initially at high relative humidity (90%) and reduced light at 20 to 27^{o}c for the first 10-15 days by keeping them under mist or covering them with clear plastic bags. After spending few days under high humidity the plants should be moved to the green house bench. Transplants should then acclimatized by gradually lowering the relative humidity over 1 to 4 weeks period. Plants are gradually moved to higher light intensities to promote vigorous growth.

OBJECTIVES

The present investigation was carried out on an important medicinal plant-*Tylophora indica.* The main objectives of the present investigation were:

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

REVIEW OF LITERATURE

The growth of all the living organisms- plants as well as animals begins with a single fertilized cell i.e. zygote. This cell is evidently the repository of all the information necessary for its subsequent growth into a multicellular, highly organized, complex but co-coordinated system. This tiny totipotent cell conceals the potential for differentiation. The differentiated somatic cells in a plant carry out specialized activities and appear to have surrendered their totipotency in the bargain.

The father of tissue culture is Gottlieb Haberlandt who in 1902 gave the idea of totipotency. He put forward the hypothesis that all plant cells are endowed with totipotency although differentiated cells don't manifest it explicitly. He went so far as to suggest that 'one could successfully cultivate artificial embryos from vegetative cells'. Haberlandt's attempt to grow vegetative cells in an artificial nutritional medium did not succeed due to lack of proper techniques and unfortunate choice of highly specialized material. Still it did open up new vistas in morphogenesis. Although the significance of Haberlandt's idea was not lost on botanists, the demonstration of totipotency consumed half a century. Haberlandt's hypothesis has by now, flowered into a vigorous discipline – "Tissue culture". This board term refers to the growth of organs, tissues and cells in an artificial media.

Although Haberlandt failed, his student Kotte (1922) and Robins (1922) partially succeeded in culturing excised roots in an artificial medium. The growth of these roots however could not be sustained for long even if they were transferred to fresh medium. White (1934) was the first to demonstrate continuous culturing using excised root-tips of tomato on a medium containing inorganic salts, sucrose, and YE. Two main thrusts in the history of tissue culture are the induction of callus and formation of somatic embryo in cell culture. Gautheret (1934) is credited with first successful attempt of callus induction on woody cambial

explants. This was followed by the formation of continuous callus cultures in carrot and tobacco by Gautheret, Nobecourt and White in 1939 independently of each other. In 1958, Steward et al. demonstrated the totipotency of higher plant cells in an unambiguous term with their success in forming somatic embryos from cultured carrot root phloem cells. During fifties rapid development of other new techniques like culturing of single isolated cells (Muir et al. 1954), growing single cell in liquid suspensions (Nickell 1956), defining chemical regulation of growth and organ formation (Skoog and Miller 1957) and recording cell division in isolated single cells (Torrey 1957) was witnessed.

The advent of *in vitro* tissue culture technique has offered a new approach to the morphogenetic investigations. Its advantage over the other techniques stem from the fact that it allows a living system to be studied under controlled environmental conditions. This enables a study of the complex biological phenomenon in its parts. These partial processes are amenable to controlled investigations. Tissue culture has now become a well established technique for culturing and studying the physiological behavior of isolated plant organs, tissues, cells, protoplasts and even organelles under precisely controlled physical and chemical conditions.

The clonal propagation of selected phenotypes is an essential step in most of the plant breeding programmes. The conventional methods of vegetative propagation like rooting of cuttings, budding, grafting and use of bulbils, suckers, rhizomes are fairly simple but have proved often cumbersome and are not applicable to all plants especially to trees and shrubs. During the last few years micropropagation technique has emerged as a promising technique for rapid and large scale propagation of selected plants. A small piece of tissue can be used to raise hundreds or thousands of plants in a continuous process. Ball (1946) successfully raised transplantable whole plants of *Lupinus* and *Tropaeolum* by culturing the shoot meristems. The potential for this work was soon realized by

Morel (1963) for rapid propagation of orchids *Cymbidium* and *Odontoglossum.* The advantage of using this method was that about 4 million genetically identical plants could be obtained from a single shoot apex. Micropropagation is a complex multistep process and the ease with which plants can be micropropagated varies from species to species.

The technique of micropropagation is based on the concept of totipotency as proposed by Haberlandt. Every cell of the plant body is totipotent i.e capable of giving rise to a new plant under proper nurture conditions. Micropropagation is now a well established technique commercialized globally for the rapid production of a number of commercially important plants like *Gladiolus, Freesia, Gerberas*, Carnations, lilies, *Chrysanthemum, Eucalyptus*, Poplar, *Dalbergia, Albizzia* and a number of important fruit trees and medicinal plants.

Multiplication by Apical & Axillary shoots:

Micropropagation through apical and axillary shoot proliferation is the most common technique for commercial mass production. The cells of apical and axillary meristems are uniformly diploid and least susceptible to genotypic changes, hence this method ensures genetic stability of the clones.

A shoot tip and an axillary bud having preformed meristems usually develop axillary shoots on a high cytokinin concentration. These axillary shoots can be subdivided into smaller clumps of shoots which in turn can develop similar cluster after subculturing on fresh media. This process can go on indefinitely and can be maintained throughout the year and thousands of plants can be raised starting from a single shoot tip or axillary bud. The multiplication rates through this technique vary with genotype and the cytokinin requirement has been extremely variable.

Patnaik and Debata(1996) developed a protocol for *in vitro* propagation of an aromatic and medicinal plant *Hemidesmus indicus* from nodal segments on MS supplemented with NAA (0.054ppm)+ K(1.5ppm). Khan and Rao (1998) reported clonal multiplication of *Syzygium alternifolium* from mature nodal segments and BAP(4ppm) +NAA (0.5ppm) were found to be best multiplication medium where the highest number of shoots per node was found within six weeks of culturing. Similarly, Ajithkumar ans Seeni (1998) achieved rapid clonal multiplication of *Aegle marmelos* by enhanced axillary bud proliferation in single node segment of a twenty five years old tree on MS medium supplemented with BAP(2.5mg/l)+ IAA (1mg/l).

Komalavalli and Rao (2000) established *in vitro* micropropagation of *Gymnema sylvestre* – a multipurpose plant. According to them the nature of the explant, seedling age, medium type, plant growth regulators, complex extracts (casein hydrolysate, coconut milk, malt extract and yeast extract) and antioxidants (activated charcoal, ascorbic acid, citric acid and polyvinylpyrrolidone) markedly influenced *in vitro* propagation.

Addition of BAP (0.3mg/l) and K (0.2mg/l) has been found to give a good response of shoot proliferation in *Withania sominifera* with a regeneration of 85% (Kulkarni et al.2000). MS medium with growth regulators such as BAP (0.5mg/l) in combination with NAA (0.01mg/l) has been reported to give optimum results in *Utleria salcifolia* (Gangaprasad 2003). The highest efficiency of shoot proliferation in *Mucuna pruriens* from the nodal segment was observed in BA (5mg/l) and NAA (0.5mg/l) in half strength MS medium (Faisal et al.2005).

Somatic embryogenesis:-

It involves the formation of a bipolar structure containing both shoot and root meristems, and developing in a manner similar to zygotic embryos. These embryoids can develop into fully functional plants under appropriate conditions. Steward et al. (1958) and Reinert (1959) firstly reported somatic embryogenesis from phloem cells of roots in *Daucus carota*. Since then embryoid formation has been reported in a wide variety of flowering plants in tissues derived from petiole, leaf, root floral parts, young & mature embryos & seedlings etc.

Pandey et al.(2002) reported somatic embryogenesis in *Podophyllum hexandrum* from cotyledonary leaves of germinated embryos on MS medium containing various concentrations and combinations of NAA (0.5-5ppm), BAP (0.5-2.5ppm) and GA3(1ppm). The embryos thus formed were placed in 3% (w/v) sodium alginate emulsion and were dropped in 1% calcium chloride solution. Beads or artificial seeds thus formed were cultured on MS + BAP (1ppm) + GA3 (2.5ppm).

An efficient method has been developed for regeneration of complete plants via somatic embryogenesis in *Corydalis yanhusuo* (Fumariaceae), an important medicinal plant, using tuber-derived callus by Sagare et al (2000). Indirect somatic embryogenesis, encapsulation and plant regeneration was achieved with the rare rhoeophytic woody medicinal plant *Rotula aquatica* (Boraginaceae) by Chithra and Martin (2005). Friable callus developed from leaf and internode explants on Murashige and Skoog (MS) medium with 2,4-D (0.45µM) was most effective for the induction of somatic embryos. Sahrawat and Chand (2002) developed an efficient plant regeneration protocol from root explants of *Psoralea corylifolia*, an endangered medicinally important herbaceous plant species belonging to the family Fabaceae.

Micropropagation through callus

Prakash et al (2002) developed a procedure for plant regeneration of *Hybanthus enneaspermus*, a rare ethnobotanical herb from the Deccan peninsula in India, through seed-derived callus. Seeds demonstrated a high induction frequency and a high yield of light-yellow friable callus on Murashige and Skoog's (MS) medium containing NAA and BA within 4 weeks of incubation. Shoot differentiation resulted when callus was transferred to MS medium supplemented with BA(8.8ppm) and NAA (2.6ppm).

Similarly, Pandey et al (2002) established a protocol for in vitro propagation of an alpine medicinal herb *Aconitum balfourii*. Axillary bud derived leaf explants formed callus on medium containing BAP(4.5ppm) and NAA(2.7ppm); simply by lowering the NAA concentration to 1.4 ppm these calli turned organogenic, and the shoots thus formed were further multiplied.

Faisal and Anis (2005) developed a protocol of high frequency shoot organogenesis and plant establishment from stem derived callus of *Tylophora indica*. Callus was developed on Murashige and Skoog (MS) medium supplemented with 2,4,5-T (10μM). Multiple shoot induction was achieved from the surface of the callus after transferring onto shoot induction medium. The highest rate (80 %) of shoot multiplication was achieved on MS medium containing kinetin (5 μM). Thomas & Philip (2005) have reported high frequency shoot organogenesis from leaf derived callus of a medicinal climber *Tylophora indica*.

Similarly, a protocol has been developed for high-frequency shoot regeneration and plant establishment of *Tylophora indica* from petiole-derived callus by Faisal & Anis (2005). Optimal callus was developed from

petiole explants on Murashige and Skoog basal medium supplemented with 2,4-dichlorophenoxyacetic acid (10 M) + thidiazuron (TDZ) (2.5 M). Adventitious shoot induction was achieved from the surface of the callus after transferring onto shoot induction medium. The highest rate (90%) of shoot multiplication was achieved on MS medium containing TDZ (2.5 M). Individual elongated shoots were rooted best on half-strength MS medium containing IBA (0.5 M).

MATERIAL AND METHODS

Choice of material

Tylophora indica, commonly known as antmool was selected as experimental material. It is a perennial, small, slender, much branched pubescent twining or climbing herb found in the sub-himalayan tract from Uttar Pradesh to Meghalaya and in the central and peninsular India. Roots are long, fleshy. Leaves are ovate-oblong to elliptic-oblong, cordate at base, thick, pubescent beneath when young, glabrous above. Flowers are minute, 2 to 3-flowered fascicles in axillary umbellate cymes.; Calyx divided nearly to the base, densely hairy outside; segments lanceolate, acute. Corolla is greenish-yellow or greenish-purple; lobes oblong, acute.

Habitat: Found in the plains, forests, and hilly slopes and outskirts of the forest. Forms dense patches in the forest in moist and humid conditions in open hill slopes and narrow valleys, also cultivated for its medicinal uses. The plant shows stunted growth in the areas with lesser rainfall.

Distribution: It is indigenous to India. The plant inhabits up to an elevation of 1,260 m in the sub-Himalayan tract and in the central and peninsular India. It also found in Eastern, North-East and Central India, Bengal and parts of South India. Except throughout plains of India, it also harbor in Ceylon, Malay island and Borneo.

Medicinal Importance: It is traditionally used as a folk remedy in certain regions of India for the treatment of bronchial asthma, inflammation), bronchitis, allergies, rheumatism and dermatitis. Apart from the above, it also seems to be a good remedy in traditional medicine as anti-psoriasis, seborrheic, anaphylactic, leucopenia and as an inhibitor of the Schultz-Dale reaction (Sarma, 1978, Sarma & Misra, 1995).

The leaves and roots are used medicinally. It is said to have laxative, expectorant, diaphoretic and purgative properties. It has also been used for the treatment of allergies, cold, dysentery, hay fever and

arthritis. It has reputation as an alternative and as a blood purifier, often used in rheumatism and syphilitic rheumatism. Root or leaf powder is used in diarrohea, dysentery and intermittent fever. It is an expectorant and administered in respiratory infections, bronchitis and whooping cough. Dried leaves are emetic, diaphoretic and expectorant. It is regarded as one of the best indigenous substitute for ipecacuanha, so it was considered as Indian ipecacuahna in the latter half of the 19th century.

Toxic effect: According to Gupta et al. (1979), it may produce some side effects like drowsiness or giddiness. Loss of taste for salt, mouth pain, upset stomach, temporary nausea and vomiting are some other side effects. Tightness in throat or chest, chest pain, skin hives, rashes, or itchy or swollen skin may occur in some cases.

Preliminary studies shows that extract of *Tylophora* is toxic only in extremely high doses; these extracts were apparently safe in the far smaller doses needed to produce a therapeutic effect.

Chemical Components: The major constituent in this plant is alkaloid Tylophorine that is responsible for a strong anti-inflammatory action. The other alkaloids include Tylophorinidine, Septicine and Isotylocrebrine.

Pharmacology: Test tube studies suggest that tylophorine is able to interfere with the action of mast cells, which are key components in the process of inflammation action. These actions seem to support its traditional use as an anti-asthmatic and anti-allergic medication by traditional healers. According to Bone (1996), the dose should not exceed 200-400 mg dried leaf powder per day or 1 to 2 ml of tincture per day for the treatment of asthma. The plant shows inhibitory effect on cellular immune response and antiallergic activity.

Glasswares

The glasswares used for culture work comprised of 6”Õ 1” borosil test tubes, 250ml, 500ml and 1000ml borosil flasks. In addition other glassware includes graduated measuring cylinder, petridishes, beakers and a range of pipettes. Before use, glasswares were thoroughly brushed with alkaline detergent teepol and then washed in running tap water. It was then treated with hot chromic acid (mixture of $K_2Cr_4O_7$ + H_2SO_4 + H_2O) followed by very thorough washing with tap water. All vessels were then inverted in a clean tray and left to dry. Copper distilled water (5-10ml) was then poured into every culture vessel which was tightly plugged. Plugs were made out of absorbent surgical cotton wrapped in muslin. Glassware was then steam sterilized in an autoclave at a pressure of 15 lb\in2 (1210C) for 15 to 20 minutes.

Culture Media

Murashige and Skoog’s (1962) medium was used as basal medium. Stock solutions of generally 8-10 times major elements, 1000 times minor elements and 100 times organic constituents were prepared. These stock solutions were stored at -4^{o}C and were mixed in desired proportions only before use. None of the stock solutions were stored for more than 15 days.

Composition of Murashige and Skoog’s medium (1962)

Ingredient	Amount (mg/l)
Major elements	
$(NH_4)NO_3$	1650
KNO_3	1900
$CaCl_2.2H_2O$	440
$MgSO_4.7H_2O$	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
Nicotinic acid	0.5
Pyridoxine HCl	0.5
Thiamine HCl	0.1
Sucrose	20000
Agar-agar	10000

Determined amounts of all the constituents except agar were mixed and volume was adjusted by distilled water. The pH of solution was adjusted to 5.8± 0.2 using 0.1N NaOH or HCl depending upon high or low.

Following are some of the supplement, which were used either singly or in combinations for induction of callus and differentiation.

1. Basal medium (BM)
2. BMS + NAA, IAA, IBA and 2,4-D(0.5-6ppm)
3. BMS + Kn or BAP (1-8ppm)

4. BMS + Kn (0.5-2ppm) + NAA, IAA or 2,4-D (0.5-6ppm)
5. BMS + BAP (0.5-4ppm) + NAA, IAA or 2,4-D(0.5-4ppm)
6. BMS + CM (15%) + NAA, IAA or 2,4D (1-6ppm)
7. BMS + CM (15%) + Kn or BAP (6ppm) + NAA, 2,4D (0.5-6ppm)

Coconut milk (liquid endosperm) when used was extracted from young coconuts and was stored at -4^{o}C. Definite aliquots of media were distributed depending upon the capacity of culture vessels. Generally 20-25ml in test tube, 50ml in 100ml flask and 100ml in 250ml flask was distributed. Test tubes and flasks were plugged with sterile cotton plugs (made of cotton wrapped in muslin cloth) and autoclaved at 15 lb/in2 (1210C) for 15 to 20 minutes. Test tubes were placed over racks that tilt the test tubes during cooling and gave slanted surface to the agar media.

Inoculation

All the experimental manipulations were carried under aseptic conditions in an inoculation chamber fitted with a bactericidal ultraviolet tube (15W, peak emission 2537A^{o}). The floor of the chamber was thoroughly scrubbed with cotton dipped in alcohol. The surface of all the vessels and other accessories such as instruments (spatula, forceps, needles and scalpel etc.), spirit lamp, matchbox, tube containing absolute alcohol etc. were also cleaned with alcohol. The fresh material to be inoculated was kept in a petridish covered with a piece of black paper in order to protect it from the harmful effects of ultraviolet rays. Alcohol was then sprayed in the chamber with the help of an atomizer. The chamber was then sterilized with ultraviolet tube kept continuously on for one hour.

Surface sterilization of inoculum

Just like media, plant tissues were disinfected before they were placed over the media. Explants like leaves, stem and nodal explant were taken from plants growing under the in vivo conditions. These were placed in different bottles and covered with net and washed for 30 minutes under running tap water to remove all the adhering dust particles and microbes from the surface. The explants were then washed with liquid detergent (teepol) for another 15 minutes and then washed properly to remove detergent. The explants were then treated with bevistin for another 20-30 minutes to remove fungus and then washed properly to remove fungicide. Under the sterile conditions, the explants were treated with 0.1% $HgCl_2$ solution for 5-10 minutes depending upon the explants. The explants like stem and nodal explants were treated with 0.1% $HgCl_2$ for 5-6 minutes. Similarly explants like leaves were treated with 0.1% $HgCl_2$ for 3-4 minutes. The explants were then thoroughly washed (4-5 washings) with sterilized distilled water to remove traces of $HgCl_2$. Fresh cuts were given to the stem explants after sterilization to remove undesirable or dead portions.

Cultural Conditions

All the cultures were maintained in an air conditioned room at a temperature of 25±4^{o}C. The source of illumination consisted of 4 feet wide fluorescent tubes (40W) and incandescent bulb (25W). The intensity of illumination was 3500 lux at the level of cultures and 12 hour light regime was followed by 12 hours of darkness.

RESULTS AND OBSERVATIONS

Shoot apices, nodal segments, leaves & stems were excised from field grown mature plant and used for experimental work.

Nodal explant culture

Fresh nodal explants 5mm in length were excised from field grown mature plant. They were surface sterilized with 0.1% $HgCl_2$ for 4-5 min & were cultured on MS medium supplemented with various growth regulators. The axillary shoot proliferation from the cultured explants was remarkably influenced by the type of concentration of the growth regulator used. Multiple shoot formation was induced from nodal explant on MS medium supplemented with different concentrations of BAP (0.5-10ppm) alone or in combination with NAA. However, nodal explants cultured on MS medium supplemented with different concentration of BAP alone showed the best results. The no. of shoots formed also varied with the concentration of BAP. 3-4 shoots were formed on lower concentration of BAP (1ppm). Highest no. of shoots per culture was obtained on MS medium supplemented with 2ppm of BAP, where nodal explant produced nearly 45-50 shoots. But with the further increase in concentration of BAP, number of shoots formed has waned to 6-11 shoots on 4ppm of BAP and 4 shoots per explant on (6-10ppm) BAP.

Figure-1 shows the formation of 4-5 multiple shoots on MS medium supplemented with BAP(2ppm) after 12 days of culturing & after 3-4 weeks a number of multiple shoots nearly 8-10 were formed on the same medium (figure-2). When nodal segments were planted on BAP supplemented medium, initially 8-10 shoots arose from the axillary position after 2 weeks of inoculation. At the same time lower cut end of the explant formed a cluster of globular structures each of which eventually developed into a shoot. In this way nearly 40-50 shoots originated from a single nodal segment. Figure-3 shows the formation of globular structures at the cut end & development of these structures into shoots is shown in

figure-4 & 5. When nodal segment bearing these structures were planted on the fresh BAP supplemented medium, they proliferated further & grew into many shoots (figure-6). The response of various growth regulators on the proliferation of shoots from nodal segments is given in Table-1 & shown in Figure-7

Table 1

Hormone conc.	**Type of explant**	**Number of shoots per explant**
BAP (0.5ppm)	Nodal	0
BAP (1ppm)	Nodal	4
BAP (2ppm)	Nodal	45-50
BAP (4ppm)	Nodal	10-11
BAP (6ppm)	Nodal	4
BAP (8ppm)	Nodal	4
BAP (10ppm)	Nodal	4
CM + BAP (0.5ppm)	Nodal	0
CM + BAP (1ppm)	Nodal	4
CM + BAP (2ppm)	Nodal	10-12
CM + BAP (4ppm)	Nodal	6-8
CM + BAP (6ppm)	Nodal	4
BAP (1ppm) + NAA (0.5ppm)	Nodal	2-3
BAP (2ppm) + NAA (0.5ppm)	Nodal	4
BAP (4ppm) + NAA (0.5ppm)	Nodal	4

Rooting of shoots

The regenerated shoots were carefully rescued from culture tubes, placed on a sterile petridish & were given the cut from basal end of cluster of shoots. Then each of these shoots was planted on rooting medium which consisted of MS medium & different concentrations of IAA, NAA & IBA for root initiation. Among the various growth regulators tested IBA (4ppm) showed the best results, where roots initiated after 15 days of culture (figure-8). Figure-9 shows complete plantlets with elongated shoot & root systems ready to be transferred to the soil.

Effects of various hormones on the rooting of excised shoots is depicted in Table-2.

Table 2

Hormone conc.	Percentage of explants forming roots	Root growth
IBA (0.5ppm)	0	-
IBA (1ppm)	0	-
IBA (2ppm)	50	++
IBA (4ppm)	80	+++
NAA (0.5ppm)	20	+
NAA (1ppm)	20	+
NAA (2ppm)	0	-
NAA (4ppm)	0	-
IAA (0.5-1ppm)	0	-
IAA (2ppm)	0	-

"-" - no response

"+" - very less rooting

"++" - average rooting

"+++" - good rooting

Transfer of plantlets to the soil

After shoot & root development, attempts were made to establish regenerated plantlets into the soil. Sufficiently rooted plantlets were transferred to small plastic glasses for hardening prior to their final transfer to the soil. The rooted plantlets were gently removed from the culture tubes keeping the roots intact by using forceps with extreme care to avoid any mechanical damage to the plantlets. Plantlets were thoroughly washed with tap water to remove any remaining agar possibly on them. Further, plantlets were given bevistin treatment (1g/l) for 10 minutes to

protect the plant from any future fungal attack. The regenerants were then transferred to plastic glasses containing soil & agropeat (2:1). Plants were thoroughly watered & kept in poly house under humidity range of 70-90% for about 10-14 days (figure-10). After this period in poly house, these plantlets were transferred to shade house in which they were kept under the humidity range of 60-70%. Nodal explants callused when planted on MS + NAA (4ppm) + K (1ppm). Like stem & leaf calli, nodal segment callus exhibited root differentiation & shoot differentiation.

Stem culture

Stem explants 3-5mm in length were excised from a mature field grown plant & were cultured on MS containing different growth regulators used either alone or in conjugation with each other.

No direct rooting or adventitious shoot formation was observed from stem explant on any of the combinations tried.

Callusing

Callusing of the stem segment occurred on MS medium supplemented with 0.5 to 4 ppm of NAA. Best growth of callus however occurred on MS + NAA (4ppm) + K (1ppm) hereby designated as NK medium, with or without CM (15%). Callusing started at the cut ends or along the entire surface after 6-8 days of culturing (figure-11) & after 3 weeks the entire segment turned into a mass of green soft & friable callus (figure-12). The callus when subcultured on NK medium proliferated further & showed sustained growth.

Stem segments also callused on 2,4-D (2ppm) or 2,4-D (2ppm) + K (1ppm), but the growth was very slow (figure-13). Good callusing also occurred when stem explants were cultured on MS + BAP (2-6ppm) but rate of growth was less as compared to NK medium. Figure-14 shows 7 weeks old stem culture callusing all over the surface on MS + BAP (2ppm). Effect of different growth regulators on callus induction from stem explant is shown in table-3.

TABLE – 3

Hormone conc.	**Percentage of explants forming callus**	**Growth**
Basal MS	0	-
MS + NAA (0.5-2ppm)	30	+
MS + NAA (4ppm)	40	++
MS + 2,4-D (2-4ppm)	40	++
MS + 2,4-D (2ppm) + K(1ppm)	40	++

MS + 2,4-D (7ppm)+ BAP (0.5ppm)	50	+++
MS + NAA (4ppm) + K(1ppm)	90	+++++
MS + IAA (0.5 – 4ppm)	0	-
MS + IAA (0.5 – 4ppm) + K(1ppm)	0	-
MS + CM + NAA (4ppm) + K (1ppm)	90	+++++
MS + CM + NAA (4ppm)	50	+++
MS + CM + NAA (2ppm)	30	+
MS + CM + IAA (2ppm) + K(1ppm)	0	-
MS + CM + 2,4-D(2ppm) + K(1ppm)	0	-

"-" - no callus
"+" - very less callus
"++" - average callus
"+++" - good callus
"+++++" - very good callus

Study of callus

Microscopic observations made on stem calli established their heterogenous nature. The cells were spheroidal, ovoid, elongated & of different sizes. Cells were having prominent nucleus & dense cytoplasm. The prominant chloroplasts were present within the cells. Plastids containing starch were also observed in some cells.

Differentiation

Xylogenesis

Two week old stem callus revealed differentiation of tracheids which occurred singly or in groups forming nodules (figure-15). They possessed sclariform thickenings on their walls (figure-16).

Rhizogenesis

Root differentiation occurred when callus was transferred to MS medium supplemented with different concentration of IBA (1-4ppm). Roots appeared 2 weeks after culturing. Initially a few roots were formed, but with further proliferation of callus more roots appeared randomly in about 80% culture. These roots were long, white or greenish in colour (figure-17).

Caulogenesis

Shoot differentiation occurred when calli from NK medium was transferred to BAP supplemented medium. 8-10 shoots differentiated from the calli on 1-2 ppm of BAP media. No loss of shoot differentiation was observed even after subsequent subculturing of calli on fresh media for several generations. Figure 18 & 19 show a few shoots & many shoot differentiated from stem calli on 2ppm of BAP after 3 & 5 weeks of culturing. The shoots elongated & grew well & developed many leaves.

Rooting of adventitious shoots

Shoots 2-3cm in length were separated and transferred to MS medium supplemented with various concentrations of NAA, IBA and IAA for rooting. On NAA callusing occurred at the base of shoots and no rooting was observed. IBA induced rooting at 4ppm concentration without formation of callus at the cut end (figure-20). No rooting was observed on IAA supplemented medium.

Leaf culture

Leaf explants 3-5mm in length were excised from mature plant & were planted on MS supplemented with various additives. On NK medium profuse growth of callus occurred from explant after 7-8 days of culturing (figure-21). Callusing occurred either at the cut ends or whole of the leaf segment callused simultaneously & within three weeks the entire explant turned into a mass of soft, green friable callus (figure-22). Leaf explant also showed callus induction on MS supplemented with 2,4-D (4ppm) + kinetin (1ppm) but the growth of callus was slow & took nearly 45 days for the explant to turn into a mass of callus. Callus formation was also observed on different concentrations of BAP (figure-23).

Study of callus

The callus obtained on NK medium was Initially soft but became solid with passage of time & thus had to be teased with needles to study its cell types. It was heterogenous in nature, since a wide range of shape & sizes of cells were observed. Majority of the cells had in them dispersed chloroplasts or abundant starch grains in them.

Xylogenesis

Histological observations on 3 week old callus revealed xylem differentiation in the form of tracheids. These were mostly grouped together in the form of nodules or nests (figure-24). The tracheids were variable in size & shape and possessed sclariform thickenings (figure-25).

Differentiation of roots

Roots were formed when callus was shifted to varying concentrations of IBA (1-4ppm). Roots appeared after 20 days of plantation on IBA medium. Initially roots formed were white bearing profuse roots hair but after further growth of roots, root hair disappeared. Figure-26 & 27 show 3 weeks & 6 weeks old leaf culture on IBA supplemented medium showing root differentiation from leaf calli.

Differentiation of shoots

Shoot differentiation occurred in 80% of cultures when callus was transferred from NK medium to BAP supplemented medium (1-4ppm). Shoot differentiation occurred after three weeks of culturing (figure-28). These shoots grew further & developed many leaves (figure-29). Shoots were excised from callus & planted on root inducing medium IBA (1-4ppm) for initiation of adventitious root at the base to form complete plantlets (figure-30).

Nodal segment

Figure 1. 4-5 shoots proliferated from nodal explant on MS + BAP (2ppm) after days of culturing.

Figure 2. 4weeks old nodal explant showing multiple shoot formation (8-10) on MS + BAP (2ppm).

Figure 3. 14 days old culture of nodal explant showing formation of globular structure at the cut ends on MS + BAP (2ppm).

Figure 4 & 5. Development of multiple shoots from globular structures on MS + BAP (2ppm) after 30 & 45 days respectively.

(1)

(2)

(3)

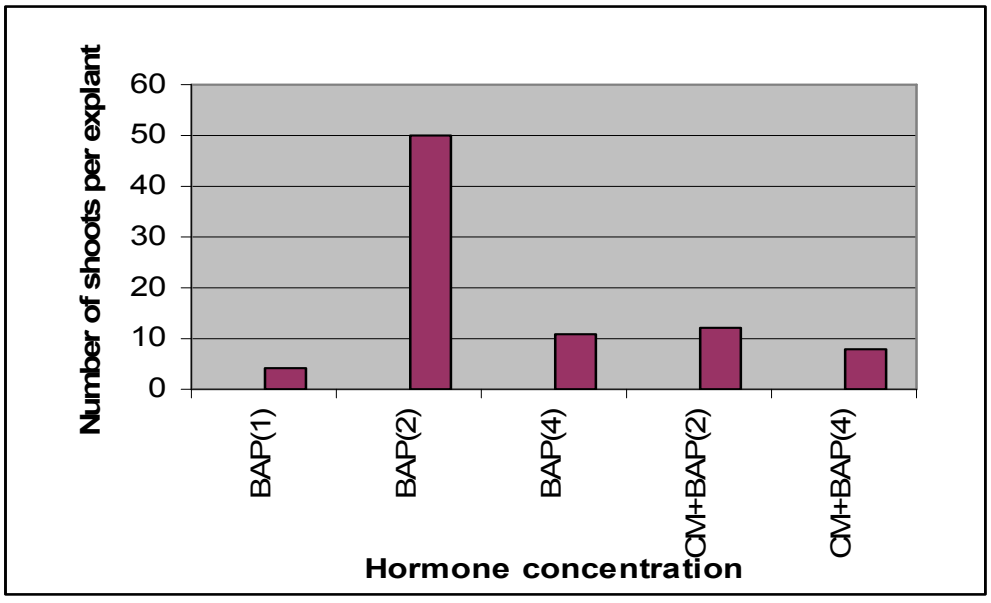
(4)

Figure 6 Multiple shoot proliferation (nearly 40) from nodal explant on MS + BAP (2ppm) after 10 weeks of culturing.

Figure 7. Effect of different concentrations of BAP & BAP + CM (15%) in MS medium on shoot proliferation from nodal explants.

(5)

(6)


Number of shoots per explant
60
50
40
30
20
10
0
BAP(1)
BAP(2)
BAP(4)
CM+BAP(2)
CM+BAP(4)
Hormone concentration

(7)

(8)

(9)

(10)

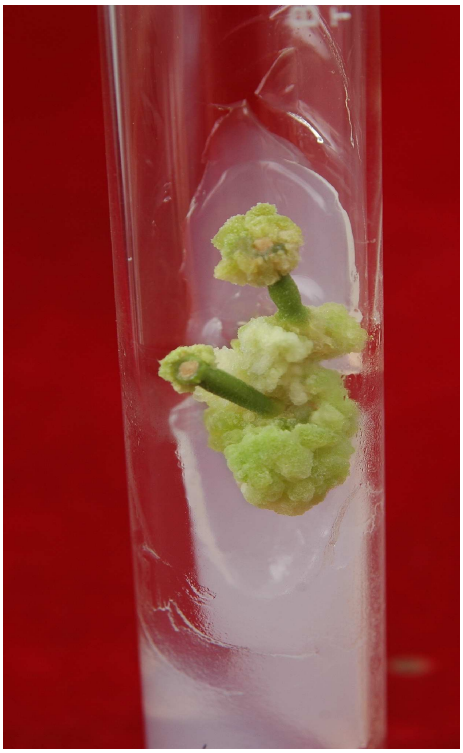
Stem culture

Figure 11. 12 days old stem culture showing callusing at the cut ends on NK medium.

Figure 12. Large mass of callus formed from stem segment on NK medium after 3 weeks of culturing.

Figure 13. 45 days old stem cultures on 2,4-D(2ppm) & 2,4-D(2ppm) + K(1ppm) respectively.

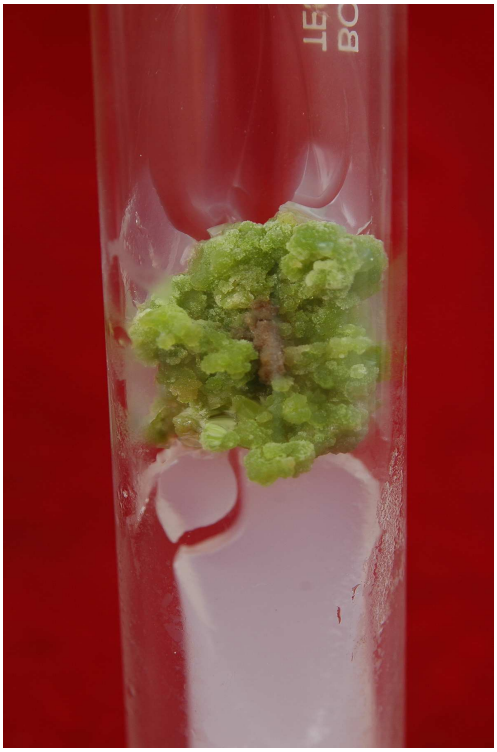
Figure 14. 7 weeks old stem culture callusing all over the surface on MS + BAP (2ppm).



(11)

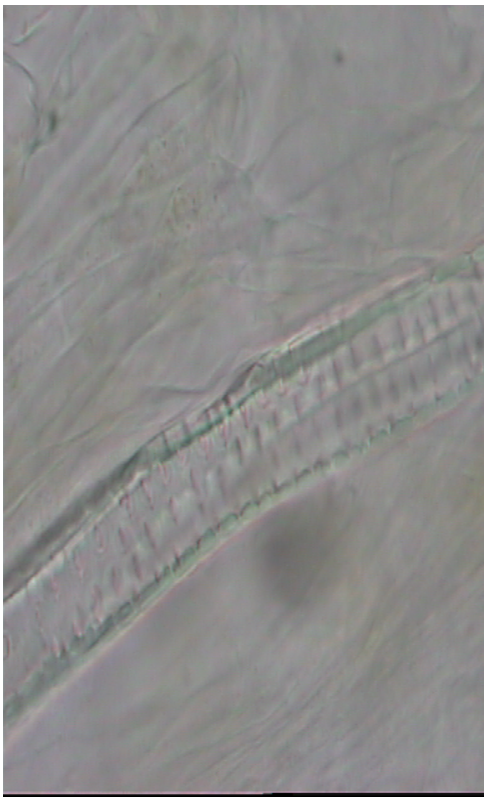
(12)

(13)



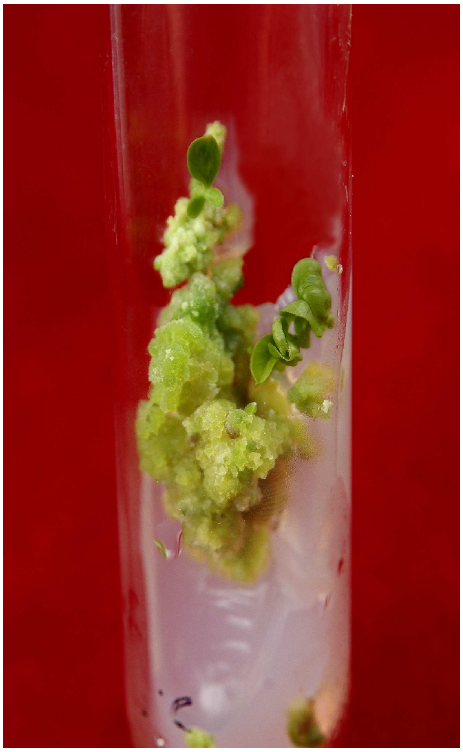
(14)

(15)



(16)

(17)



(18)

Figure 19. Differentiated shoots from callus growing further and developing many leaves.

Figure 20. Adventitious root formation at the lower end of excised shoots after 4 weeks of culturing.

(19)

(20)

Leaf culture

Figure 21. Leaf explants callusing at the cut ends on MS + NAA (4ppm) + K (1ppm).

Figure 22. Formation of green friable callus from NK medium after 4 weeks of culturing.

Figure 23. 45 days old leaf culture showing callus formation on MS + 2,4-D (4ppm) + K (1ppm).

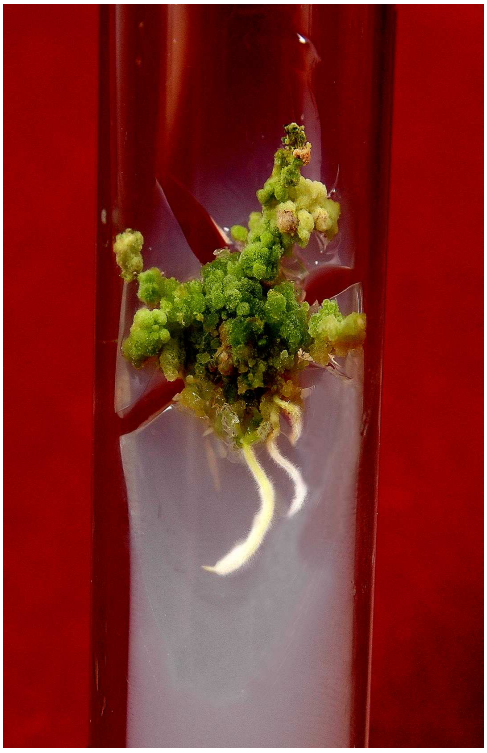
(21)

(22)

(23)

(24)

(25)



(26)

(27)

Figure 28 & 29. Showing shoot differentiation when leaf callus was transferred from NK to BAP supplemented medium.

Figure 30. Adventitious root formation on MS + IBA (4ppm) on differentiated shoot after 4 weeks of culturing.

(28)

(29)

(30)

DISCUSSION

The present investigation was undertaken on an important medicinal plant- *Tylophora indica* with a view to develop an efficient, reliable & reproducible protocol for its clonal propagation under *in vitro* conditions.

Proliferation of axillary shoots was induced from nodal segments collected from field grown mature plant. Multiple shoot formation from nodal explants occurred on MS medium supplemented with different concentrations of BAP alone or in combination with low concentration of NAA. Best results were, however , obtained on MS medium supplemented with 2ppm of BAP, where 40-50 shoots could be regenerated from a single nodal segment. Initially only 4-5 shoot buds arose per explant after 10-12 days but the number gradually increased up to 10-12 after 3-4 weeks on MS medium supplemented with 2 mg/l BAP. These shoots were healthy and sturdy in their organization.

For shoot proliferation, cytokinins are one of the most important factors affecting the response (Lane 1979, Stolz 1979, Bhojwan 1980, Garland & Stolz 1981). A wide range of cytokinins like Kinetin, BAP, 2-ip & Zeatin have been employed in shoot proliferation (Bhojwani & Razdan 1983). A number of plants such as Blueberries (Cohen 1980), Garlic (Bhojwani 1980), Annatto (Sharon & D'Souza 2000) & *Gardenia jasminoides* (Chuenboonngarm 2001) have been successfully multiplied by 2-ip. However, a wider survey of literature suggests hat BAP is the most reliable & effective cytokinin. Sudhersan et al (2001) reported multiple shoot proliferation from the shoot tip & nodal explants of *Ziziphu*s on MS medium supplemented with 0.01-0.1 mg/l BAP. On the other hand Mao et al (1995) documented that higher concentration of BAP was necessary to induce highest number of shoot regeneration through nodal segments of *Clerodendrum*. The caulogenic effect of BAP, observed in the present study, is in consonance with other reports (Kumar & Kumar 1997, Gurel & Gulsen 1998, Khan et al 1998, Shahzad et al 2002, Batra et al 2002). It was observed in the present material, that with increase in the BAP concentration from 2mg/l onwards the number of shoots per node decreased considerably. Similar observation was made by Batra

et al (2002) on the micropropagation of Neem & by Nagakubo et al 1997 on shoot regeneration frequencies in *Allium sativum*.

Induction & development of roots at the bases of *in vitro* grown shoots is an essential & indispensable step to establish tissue culture derived plantlets to the soil. Shoots thus formed were excised & subjected to rooting on MS medium supplemented with auxins. Amongst some of the auxins (IAA, IBA/NAA) tried for rooting, IBA proved best for the induction of adventitious roots from the base of regenerated shoots. Use of NAA resulted in callusing at the base of shoots whereas addition of IAA did not cause rooting. Similarly, the essentiality of IBA for root induction has been demonstrated in *Azadirachta* (Batra et al 2002) & *Mentha* (shahzad et al 2002).

The regenerants were successfully transferred to pots for hardening & acclimatization. About 80% of the total regenerants survived after 8-10 weeks of their transfer to the soil. The present study seems to be the first an *in vitro* propagation of the species- *Tylophora indica* via multiple shoot formation from nodal segments & subsequent rooting. Thus the micropropagation protocol developed for *Tylophora indica* can be successfully applied for large scale multiplication.

In the present work, the medium was supplemented with various concentrations of different growth hormones for the induction of callus from various explants taken from field grown mature plants. Growth hormones NAA &2,4-D alone could initiate callusing from stem, leaf & nodal segments but callus was slow growing. Addition of kinetin considerably enhanced the callus growth when used in conjunction with auxins. Synergistic combination of kinetin (1ppm) with NAA (4ppm) hereby designated as NK medium gave the best results. Callus was also obtained on MS medium containing combination of CM & NAA, but time taken was more. Similarly good callusing also occurred on MS + BAP (2-6ppm) but rate of growth was slow as compared to NK medium. The callus raised on NK medium showed good & sustainable growth on subculturing.

Faisal & Anis (2005) reported callus induction from stem & petiole segments of *Tylophora indica* on MS medium supplemented with 2,4,5-T (10µm) and 2,4-D & TDZ respectively.

The spectrum of induced differentiation from calli was wide & included xylogenesis, rhizogenesis & caulogenesis from calli raised from different explants.

Rhizogenesis was obtained when callus was transferred from NK medium to IBA supplemented medium. Likewise, Pandey et al (2002) achieved rooting from callus of *Aconitum balfourii* when it was planted on different concentrations of IBA.

In the present study, no differentiation of shoots could occur on NK medium but when calli was transferred to BAP supplemented medium (1-2ppm), a good number of shoots could be differentiated from the calli. No loss of shoot differentiation was observed even after subsequent subculturing of calli on fresh media for several generations.

Faisal & Anees (2005) have achieved multiple shoot induction from the surface of callus on MS containing 5µM kinetin whereas Thomas & Philip (2005) achieved plantlet regeneration from the callus on 8µM thidiazuron at which shoot regeneration was obtained from 100% of cultures with an average of 66.7 shoots per culture.

In conclusion, the present work which is an attempt to investigate the dedifferentiation & redifferentiation responses of cells of various organs of *Tylophora indica* to varied & diverse chemical milieu corroborates the concept of totipotency as proposed by Haberlandt (1902) & supports the contention of Mehra & Mehra (1971) that with proper nurture almost any living cell of plant body, be it a part of the root, hypocotyls, stem, leaf or flower can be induced to divide & mobilize & express its hidden potencies in varying manners out of its totipotent genome.

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*Not consulted in original.

ABBREVIATIONS

A^o	Angstorm
BAP	Benzylaminopurine
BMS	Basal Murashige and Skoog"s medium
^oC	Degree Celsius
CM	Coconut milk
2,4-D	2,4-dichloro phenoxy acetic acid
2,4,5-D	2,4,5-trichloro phenoxy acetic acid
IAA	Indole-3-acetic acid
IBA	Indole butyric acid
2-ip	2-isopentenyl adenine
K	Kinetin
NAA	Naphthalene acetic acid
ppm	Parts per million
YE	Yeast extract
W	Watt
TDZ	Thidiazuron
NK media	NAA (4ppm) + K (1ppm)