CLONING OF *TYLOPHORA INDICA* THROUGH FORCED AXILLARY BRANCHING AND *DE NOVO* ADVENTITIOUS SHOOT FORMATION

Thesis submitted in

Partial fulfilment for the award of Degree of

Master of Science in Biotechnology

By

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CANDIDATE'S DECLARATION

I hereby declare that the work presented in thesis entitled, "Cloning of *Tylophora indica* through forced axillary branching and *de novo* adventitious shoot formation" in partial fulfilment for the award of degree of Master of Science in Biotechnology and Environment Sciences, Thapar University, Patiala, is an authentic record of my own work done during the period of six months from January 2012 to June 2012, under the guidance of Dr. Manju Anand, Assistant Professor, 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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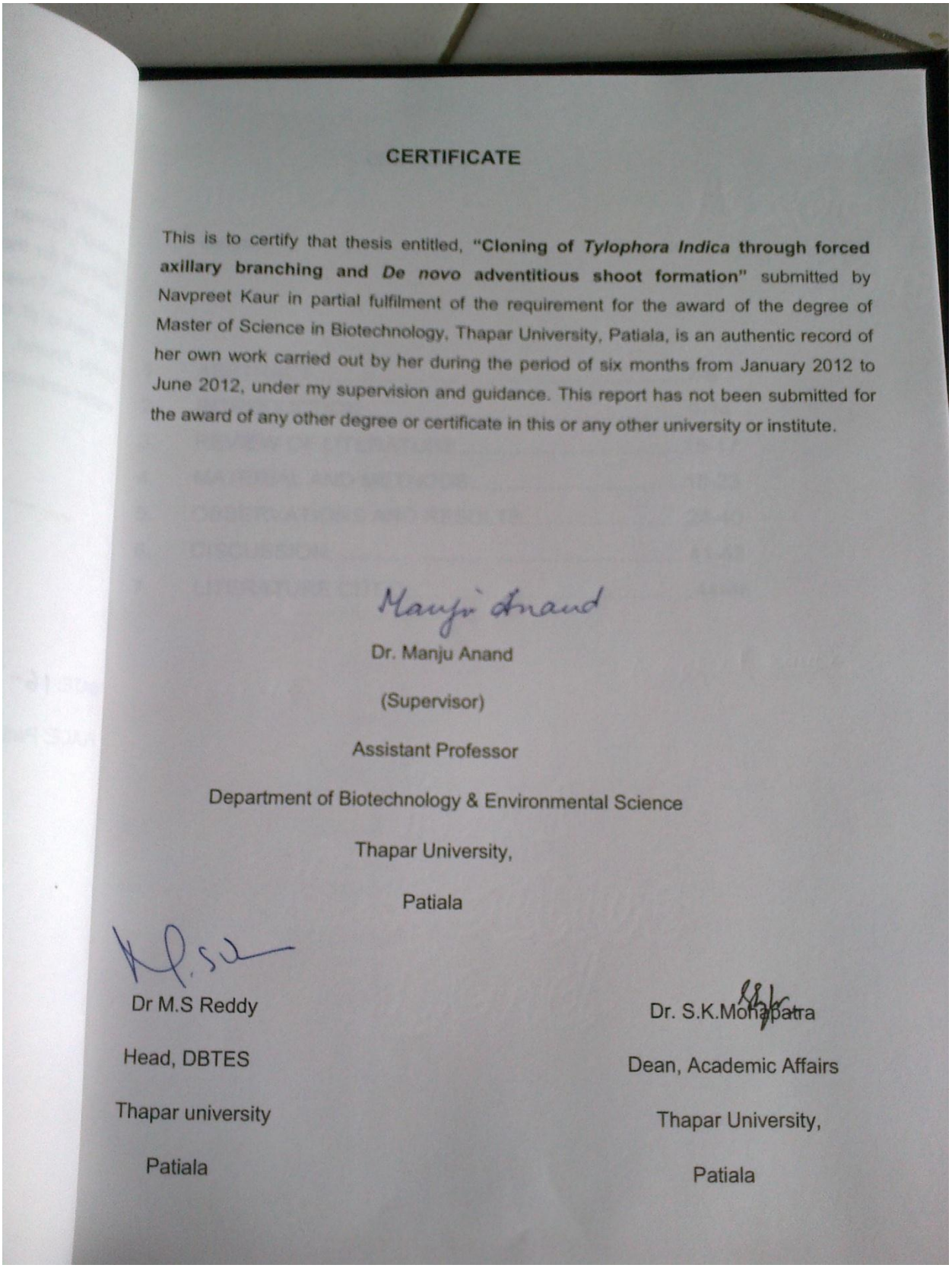
CERTIFICATE



CERTIFICATE

This is to certify that thesis entitled, **"Cloning of *Tylophora Indica* through forced axillary branching and *De novo* adventitious shoot formation"** submitted by Navpreet Kaur in partial fulfilment of the requirement for the award of the degree of Master of Science in Biotechnology, Thapar University, Patiala, is an authentic record of her own work carried out by her during the period of six months from January 2012 to June 2012, under my supervision and guidance. This 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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June, 2012 NAVPREET KAUR

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ABBREVIATIONS

Å	Angstrom
BAP	Benzylaminopurine
BMS	Basal Murashige and Skoog's medium
MS	Murashige and Skoog's medium
℃	Degree Celsius
NAA	Naphthalene acetic acid
2, 4-D	2, 4-dichlorophenoxy acetic acid
IAA	Indole 3-acetic acid
IBA	Indole 3-butyric acid
2-ip	2-isopentenyl adenine
Kn	Kinetin
Zn	Zeatin
AS	Adenine sulphate
TDZ	Thidiazuron
µM	micro molar
W	watt

ABSTRACT

The present investigation was carried out on an important medicinal plant *Tylophora indica* belonging to family Asclepiadaceae with an aim to establish an efficient and reproducible *micropropagation* protocol for its mass production. The different vegetative parts i.e. leaf, nodal segment and shoot apices taken from an elite, field grown, 4 years-old-plant and thereafter planted on variously supplemented Murashige and Skoog's medium for *de novo* adventitious shoot induction and multiple shoot proliferation.

The plant exhibited high degree of propensity of *de novo* shoot proliferation directly from leaf segments on MS medium supplemented with BAP (8.8 µM) either alone or in combination with adenine sulphate (1.35µM). Nodular meristemoids differentiated from the cut ends of leaf lamina after 8-10 days of culturing and covered the whole surface within 3-4 weeks. Eventually these meristemoids developed into green leafy shoots in nearly 80% of cultures. Initially, fewer shoots were formed but number increased further to 55-60 shoots per flask on subsequent sub culturing in about 90% of the cultures.

Tylophora indica exhibited high degree of multiple shoot proliferation from nodal segments and shoot apices taken from *in vivo* plants. Bud break and axillary shoot proliferation from nodal segments occurred on MS supplemented with 17.6-µM 6-benzyl adenine forming nearly 20-25 shoots/explant after 6 weeks. However, prolific multiple shoot induction from nodal explants occurred on MS medium supplemented with 22 µM 6-benzyladenine in conjunction with α- naphthalene acetic acid (3.67 µM) and L-ascorbic acid (8.4 µM), where 45-50 shoots regenerated from single axillary bud after 5-6 weeks of culturing. Shoot apices also exhibited multiple shoot proliferation when cultured on MS medium supplemented with BAP (17.6-22.0µM) either alone or in combination with NAA (3.6µM) + L-ascorbic acid (8.4µM), where 20-25 shoots were obtained after 6-7 weeks of culturing.

The regenerated shoots were rescued from the culture vessels and transferred onto half strength MS medium, full strength MS medium and MS medium supplemented with different concentrations of IAA, NAA and IBA for root induction. The best rooting response (90%) was observed on auxin free half strength MS medium whereas addition of IBA to full strength MS medium also showed good results. Rooted plantlets were then transferred to moist cotton jars covered with plastic bags for initial acclimatization and were kept in growth room for 12-15

days. They were then transferred to the potting mixture of soil: vermicompost (1:1) and plants with newly formed leaves were shifted to green house bench with 90% survival rate.

Introduction

Medicinal Plants: an overview

Medicinal plants have been an integral part of life in various regional communities for food and drugs both. Medicinal plants are largely used by all divisions of the population either directly as folk medications or indirectly in the preparation of recent pharmaceuticals. Herbal medicine is the oldest form of health care known to man and over 9000 herbs have known medicinal applications in various cultures and countries (Farnsworth and Soejarto, 1985). The use and Knowledge of medicinal plants is evidenced through ancient records of all major systems of medicine such as Ayurveda, Unani, Chinese medicine and Japanese Kampo. India has more than 3,000 years of medicinal heritage based on medicinal plants. Medicinal plants are an important source of bioactive compounds for the pharmaceutical industry and traditional medicine.

According to World Health Organization (WHO), about 80% of the population living in developing countries still use traditional medicines derived from plants for their primary health care needs. Medicinal plants form the resource base for rapidly growing pharmaceutical industry with 25% of drugs derived directly from the plants and many other compounds isolated as synthetic analogues. These herbal drugs are used in pharmaceuticals, neutraceuticals, and cosmetics and as food supplements. Allopathic medicine too owes a tremendous debt to medicinal plants, as one in four prescriptions, filled in a country like United States is either a synthesized form or derived from plant material (Srivastava *et al*., 1995). Medicinal plants have gained the faith during the past few years in view of their lesser side effects as compared to allopathic medicine. Drugs obtained from plants are believed to be much safer and exhibit a remarkable efficacy in the treatments of various ailments (Siddique *et al.,* 1995).

Over the past few years, demand for medicinal plants has increased in both developed and developing countries due to an escalating faith in herbal medicines. Unfortunately, rapid industrialization and urbanization have led to overexploitation and loss of valuable natural resources, including medicinally important plants. Many species have been subjected to extensive and unregulated collection which is putting a heavy strain on existing resources and posing a definite threat to the genetic stock and biodiversity of medicinal plants, causing

a number of plants to be either threatened or included in the endangered category. (Arora and Bhojwani, 1989; Purohit et al., 1994; Sudha and Seeni, 1994).

Trade of medicinal plants:

The global herbal product markets are mainly in Europe and North America which together account for 63% of the world market. The European market for herbal remedies accounts for 45% of the global market and stood at US $ 7.5 billion in 1997. Germany and France are the most established markets with a share of 22% and 11% in Europe respectively. China is the major exporter of traditional medicine to the world market.

Global market for herbal products, which includes medicines, health supplements, herbal beauty and toiletry products are estimated at around US $ 62 billion. The world Health Organization (WHO) has estimated that the present demand for medicinal plants is approximately US $ 14 billion per year. World demand for herbal products has been growing at a rate of 15%-25% per annum and according to an estimate of WHO, the demand for medicinal plants is likely to increase to US $ 5 trillion in 2050. In India, the medicinal plant related trade is estimated to be approximately US $ 1 billion per year. It is estimated that Europe annually imports about 400,000 tones of medicinal plant material with an average value of US $ 1 billion from Africa and Asia. It is found that by 1990, around 2223 major companies world wide were reportedly screening plants for new leads and more than 2000 companies were marketing herbal medicines alone in the Europe (Tewari, 1996). Medicinal plants, thus, offer remarkable opportunities to generate income and employment and to boost country's economy through foreign exchange.

Status of Medicinal Plants in India:

India is a rich country in terms of its biodiversity. India is among 12 biodiverse countries of the world having 16 agro climatic zones, 10 vegetative zones and 15 biotic provinces (Samy and Gopalakrishnakone, 2007). There are about 45,000 plant species with continental hotspots in the region of Eastern Himalayas, Western Ghats and Andaman and Nicobar islands. India is one of the leading countries in Asia in terms of the wealth of traditional knowledge systems related to herbal medicine and employs a large number of plant species, which includes Ayurveda (2000 species), Siddha (1121 species), Unani (751 species) and Tibetan (337 species) (Kala, 2002). More than 70% Indians or 1.1 billion population use these herbal-based formulations regularly as spices, home remedies and health foods as

these are non-narcotic and almost without any side effects. The northern part of the country harbors a great variety of medicinal plants because of the majestic Himalayan range. The Himalayan region is a reservoir of a large number of medicinal and aromatic plants (MAPs) and designated as one of the global biodiversity hotspots, where ecological, phyto-geographical and evolutionary factors favour high species diversity. The Himalayan region occupies only 15% of the country's geographical area, it accounts for about 30% of the endemic species found in the Indian subcontinent. This region alone supports about 18,440 species of plants of which about 45% are having medicinal potential. Though India has a rich biodiversity, about 90% of the medicinal plants used by industries are collected from the wild with 70% of the plant collection involving destructive harvesting because of the use of plant parts like root, bark, wood, stem and the whole plant. The growing demand is putting a heavy strain on existing resources, which pose a definite threat to the genetic stock and biodiversity of medicinal plants, causing a number of plants to be either threatened or included in the endangered category. The assessments done so far for the prioritized native medicinal species, have resulted in assignment of IUCN red list status to nearly 250 plant species with 44 species being critically endangered, 113 endangered and 87 vulnerable (Ved and Tandon, 1998 and Ved and Goraya, 2007).

In a wider context, there is a growing demand for plant based medicines, heath products, pharmaceuticals, food supplements, cosmetics etc. in the national and international markets. Conservation and sustainable use of medicinal plants are issues on which immediate focus is required in the context of conserving biodiversity and promoting and maintaining the health of local communities, besides generating productive employment for the poor with the objective of poverty alleviation in tribal and rural areas. (Kumar *et al.,* 2010).

Medicinal Plants and Plant Tissue Culture

In view of the growing world population, increasing anthropogenic activities and rapidly eroding natural ecosystems, the natural habitats for a number of medicinal plant species are dwindling. The rising demand of plant-based drugs is creating heavy pressure on selected, high valued medicinal plants populations due to over harvesting. To cope up with this alarming situation, the recent advances in Biotechnology especially Plant Tissue Culture have come as a boon. The identification of active principles and their molecular targets from traditional medicine provides an enormous opportunity for drug development. Using modern biotechnology, plants with specific chemical compositions can be mass propagated and

genetically improved for the extraction of bulk active pharmaceuticals. Although there has been significant progress in the use of biotechnology using tissue culture and genetic transformation to investigate and alter pathways for the biosynthesis of target metabolites, there are many challenges involved in bringing plants from the laboratory to successful commercial cultivation.

Most of the medicinal plants either do not produce seeds are too small and do not germinate in soil. Thus mass propagation of disease free planting material is the general problem. Moreover, sexually propagated plants demonstrate a high degree of heterogeneity since their seed progeny are not true-to-type unless they have been derived from inbred lines. As a result, plants raised through seeds show great variations in growth, habit and yield. Likewise, majority of the medicinal plants are not amendable to vegetative propagation by cutting or grafting.

In recent years, tissue culture has emerged as a promising technique to obtain genetically pure elites rather than having indifferent populations under *in vitro* conditions. *In vitro* propagation from very small plant parts also called micropropagation is in fact the miniature version of conventional propagation, which is carried out under aseptic conditions. Micropropagation holds a significant promise for true-to-type, rapid and mass multiplication under disease free conditions. Plants raised through micropropagation are:

- Of uniform quality and produce uniformly superior seeds.
- Have higher rate of multiplication.
- Are diseases free and show improved vigor and quality.
- Can be produced much more rapidly and throughout the year irrespective of seasons.
- For the identification and production of clones with desired characteristics.
- For the production of secondary metabolites.
- New and improved genetically engineered plants can be produced.
- For the conservation of threatened plant species

TECHNIQUES OF MICROPROPAGATION:

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

1. Enhanced axillary shoot proliferation.

Micropropagation through apical and axillary shoot proliferation is the most common 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. *De novo* formation of adventitious shoots.

New adventitious shoots can develop either directly from the explants like root, stem, petiole, leaf lamina and flower parts etc.

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 embryogenesis**

It involves the formation of bipolar somatic embryos having root and shoot axis, which can develop into fully functional plants under appropriate conditions.

Stages of micropropagation:

Micropropagation involves 4 definite stages. These are as follow:

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

Stage 1: Initiation and establishment of aseptic culture. (Main steps: explants isolation, surface sterilization and establishment of explants on appropriate culture medium).

Stage 2: Shoot multiplication or somatic embryo formation using a defined culture medium.

Stage 3: Rooting of regenerated shoots and germination of somatic embryos. Shoots are separated manually from clusters and transferred to a rooting medium supplemented with auxin or on a medium having low salt concentration and/or low reduced sugar level.

Stage 4: Transfer of plantlets to natural environment through acclimatization or hardening. Hardening of plantlets imparts some tolerance to moisture stress and a shift from heterotrophic to autotrophic nutrition. During hardening, plantlets develop cuticle and their stomata start functioning. Hardened plantlets are then transferred to glass or polyhouse under normal environmental conditions.

Rationale and objectives:

The present investigation was carried out on an important medicinal plant- *Tylophora indica***.** This important medicinal plant is rapidly disappearing and is now listed as one of the plant species in India vulnerable to extinction. *Tylophora* is normally propagated through seeds, but seeds are too small and have low seed viability and germination (Thomas and Philip, 2005). Being cross-pollinated, the seed progenies show great heterozygosity and may not be suitable for large-scale propagation of this plant species. Likewise, the plant is not amenable to vegetative propagation through cuttings or grafting, thus, limiting multiplication of desired cultivars. In view of the difficulties in the propagation of this medicinal plant, the present study was conducted with the following objectives:

- To develop a reliable protocol for rapid and mass propagation through forced axillary branching and de novo adventitious shoot formation.
- To obtain genetically pure elites rather than having indifferent populations under *in vitro* conditions

Review of literature

The clonal propagation of selected phenotypes is an essential step in most of the plant breeding programmes. Micropropagation enables large-scale production of therapeutically high value taxa for commercialization and sustainable utilization in the industrial sector. *Tylophora indica* (Burm.f.) Merrill previously called as *Tylophora asthematica,* a member of Asclepiadaceae is an important indigenous medicinal plant. *In vitro* propagation also called micropropagation is in fact the miniature version of clonal propagation, which is carried out under *in vitro* conditions. The technique of micropropagation is based on the concept of totipotency as proposed by Haberlandt who suggested that every cell of the plant body is totipotent i.e. capable of giving rise to 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.

Micropropagation can be achieved by any of the three approaches:

1) Enhanced axillary shoot proliferation.

2) *De novo* adventitious shoot formation.

3) Somatic embryogenesis.

Multiplication by apical and axillary shoots:

Micropropagation through apical and axillary shoot proliferation is most common technique for commercial mass production. A shoot tip or an axillary bud has preformed meristem, which develops axillary shoots on a high cytokinin concentration. These axillary shoots can be subdivided into smaller clumps of shoots, which in turn can develop similar clusters after sub culturing on fresh media. This process can go on indefinitely and can be maintained throughout the year and a large number of plants can be raised starting from a single shoot tip or an axillary bud. This method ensures genetic stability of the clones as the cells of meristems are uniformly diploid and are least susceptible to genotypic changes.

The multiplication rates through this technique vary with genotype and the cytokinin requirement has been extremely variable. Among different cytokinins, BAP and Kn when used either alone or in combination with lower concentration of auxins have been very effective in inducing sprouting of axillary buds in several medicinal plant species like *Syzgium*

alternifolium (Khan and Rao, 1998), *Mentha piperita* (Sunandakumari et al., 2003), *Rawolfia serpentina* (Baksha et al., 2007), *Ceropegia intermedia* (Karuppusamy et al., 2007), *Stevia rebaudiana* (Debnath, 2008, Anbazhagan *et al*., 2010), *Withania somnifera* (Fatima and Anis, 2010), *Ocimum gratissimum* (Saha et al., 2011), and *Cocculus hirsutus* (Meena *et al*., 2012).

Sharma and Chandel (1992) developed a protocol for *in vitro* multiplication of *Tylophora indica* from axillary buds on MS medium containing BAP (22µM), NAA (3.6µM) and ascorbic acid (100mg/l). Addition of ascorbic acid was reported to be essential to induce sprouting of axillary buds. Similar set of combination was used by Faisal et al., (2007) who obtained highest number of shoots (8.6± 0.71) via enhanced axillary bud proliferation from nodal explants in *Tylophora indica.* Rana and Rani (2010) reported high frequency bud break (85%) and multiple shoot formation from nodal segments explanted between September through November and cultured on MS medium supplemented with 8.8µM BAP.

De novo adventitious shoot formation

De novo formation of adventitious shoots through direct organogenesis 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 embryoid phases and significantly reducing the total no. of stages in culture. New adventitious shoots can develop directly from the explants like root, stem, petiole, leaf lamina and floral parts. Many medicinal plants like *Withania somnifera* (Kulkarni *et al.,* 2000), *Ophirriza prostrate* (Beegum *et al.,* 2006), *Pyrethrum* (Hedayat *et al.,* 2009) *Cassia corylifolia* (Siddique *et al.,* 2010), Embelia ribes (Annapurna and Rathore, 2010), Psorelea corylifolia (Baskaran and Jayabalan, 2010) have been successfully propagated *in vitro* by adventitious shoot formation.

A rapid *in vitro* propagation system has been developed by Bera and Roy (1993) who reported formation of multiple adventitious shoot buds from the mature leaf explants of *Tylophora indica* on MS + BAP (22µM) + adenine sulphate (1.35µM). Chaudhari *et al.,* 2004 reported the formation of organogenic nodular meristemoids from root explants of *Tylophora indica* cultured on MS medium supplemented with BAP or 2-ip. These meristemoids showed two types of organogenic responses when maintained on induction medium leading to direct shoot formation in 42% of cultures and somatic embryogenesis in 39% of explants. Kaur *et al.,* 2011b,c reported high frequency *de novo* adventitious shoot formation from stem and root

explants of *T.indica* when cultured on 8.8μM BAP whereas leaf explants gave better results when 9.84μM BAP was used in conjunction with 1.35μM adenine sulphate (Kaur *et al.,* 2011a).

Somatic embryogenesis

Somatic embryogenesis involves the formation of bipolar structure containing both shoot and root axis and developing in the same manner similar to zygotic embryo. These embryo like structures have been variously designated as accessory embryos, adventives embryo or embryoids. 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 root in *Daucus carota.*

Embryoid formation has been reported in tissue and organ culture of a number of plant species derived from leaf, petiole, root, floral parts and nucellar tissue of *Pimpinella tirupatiensis* (Prakash et al., 2001), *ceropegia candelabrum* (Beena and Martin, 2003), *Syngonium podophyllum* (Zhang et al., 2005), *Rotula aquatica* (Chithra and Martin, 2005), *Rauvolfia serpentina* (Singh et al., 2009), *Withania somnifera* (Sharma et al., 2010), *Chrysanthemum* (Mani and Senthil, 2011) and many more medicinal plants.

Manjula *et al.,* 2000 reported somatic embryogenesis from leaf callus of *Tylophora indica* on MS supplemented with BAP (4.4-8.8μM) along with IAA. Jayanthi and Mandal, 2001 induced embryogenic callus from leaf explants of T.indica on MS medium supplemented with 2,4-D (9μM) and kn (0.05μM) and further maturation and development of embryos occurred on different concentrations of BAP and 2-ip. Chandrasekhar *et al.,* 2006 reported somatic embryogenesis from *Tylophora indica* leaves on MS medium supplemented with thidiazuron (TDZ) in addition to 2,4-dichlorophenoxy acetic acid (2,4-D), particularly 0.5μM TDZ along with 1.5 μM 2,4-D was very effective in inducing somatic embryos.

Material and methods

Choice of material:

Tylophora indica (Burm. f.) Merrill (family Asclepiadaceae) commonly known as "Antmool" or "Dama Bel" was selected as an experimental material. This indigenous medicinal plant has multifarious uses and has been used traditionally in the treatment of certain ailments particularly bronchial asthma, bronchitis, allergies, rheumatism and dermatitis.

Morphology:

It is a perennial branched climber having cylindrical, twinning stem with long fleshy roots. Leaves are ovate-oblong to elliptic oblong, green in color with smooth surface (fig.1). Flowers are bisexual and occur in many colors like green, yellow and purple, arranged in umbellate cymes. Corolla has oblong acute lobes and is greenish-yellow or greenish purple in colour (fig. 2).

Fig. 1

Fig. 2

Habitat and distribution

Tylophora indica grows in the plains, forests, hilly slopes and outskirts of the forests with optimum temperature range of 5-35^{o}C and annual rainfall of 500-2500 mm. Plant forms dense patches in well drained soils under moist and humid conditions,but shows stunted growth in the areas with lesser rainfall (Nadkarni, 1976). Plant is indigenous to India native to the plains and hill forests of Eastern Southern India up to an altitude of 1260 m. It is found in Sub Himalayan tract from Uttar Pradesh to Meghalaya and in Central and Penninsular India. It also harbors in Ceylon, Malay Island and Borneo (Kirtikar and Basu, 1975).

Active ingredients:

The leaves and roots of this medicinally important plant contain active alkaloids such as tylophorine ($C_{24}H_{27}O_4N$), tylophorinine ($C_{23}H_{25}O_4N$) and tylophrinidine ($C_{22}H_{22}O_4N$) (Mulchandani 1971 and Bhutani *et al*.,1985). The non-alkaloidal compounds isolated from *Tylophora indica* are kaempferol, quercetin, α- and β- amyrins, tetratriacontanol, octaosanyl octacosanoate, sigmasterol and β-sitosetrol (Gupta *et al*, 2010).

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 (Kirtikar and Basu, 1975 and Varrier *et al*., 1994). The leaves and the roots are used medicinally as they have expectorant, diaphoretic and purgative properties. It has reputation as a blood purifier. Root or leaf power is used in diarrohea, dysentery and intermittent fever. It is an expectorant and administered in respiratory infections, bronchitis and whooping cough. 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 effects:

According to Gupta et al. (1979), it may produce some side effects like drowsiness and 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 showed that the extracts

of *Tylophora* are toxic only in extremely high doses while the smaller doses are safe and produce therapeutic effect.

Pharmacology:

In test tube studies, tylophorine is able to interfere with the action of mast cells, which are the 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 power per day or 1 to 2 ml of tincture per day for the treatment of asthma. The plant shows inhibitory effects on cellular immune response and antiallergic activity.

Glassware:

The glassware used for experimental work comprised of conical flasks (100ml, 150ml, 250ml, 500ml and 1000ml), culture tubes (25×125mm) and culture bottles (8×3inches). In addition, other glassware included graduated measuring cylinders, Petri dishes, beakers and a range of pipettes (1ml, 2ml, 5ml and 10ml). Before use all the glassware was subjected to chromic acid solution (mixture of $K_2Cr_4O_7$ + H_2SO_4 + H_2O) followed by thorough washing with tap water. All the vessels were washed with detergent and then cleaned with running tap water and oven dried after rinsing them with distilled water.

Culture media:

Murashige and Skoog's (1962) medium was used as basal medium. Stock solutions of generally 4 times major elements, 100 times minor elements and 10 times organic constituents were prepared. These stock solutions were stored at 4^0C 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 element:	
$(NH_4)\ NO_3$	1650
KNO_3	1900
$CaCl_2 2H_2O$	490
$MgSO_4.7H_2O$	370
KH_2PO_4	170
$FeSO_2.4H_2O$*	27.8
Na_2EDTA*	37.3
Minor elements:	
$MnSO_4 4H_2O$	2.3
$ZnSO_4.7H_2O$	8.6
H_3BO_3	6.2
KI	8.3
$Na_2MoO_4.2H_2O$	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	20,000
Agar-agar	10000

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

Definite amounts of all the constituents except agar were mixed and volume was adjusted by distilled water. The pH of the medium was adjusted to 5.8 ± 0.2 using 0.1N NaOH or HCl depending upon high and low. Definite aliquots of medium were distributed depending upon the capacity of culture vessels. Generally 100ml medium was dispersed in each conical flask (250 ml), 20ml in each culture tube and 40 ml in culture bottles (8×3”). Vessels were plugged with non-absorbent cotton wrapped in muslin cloth and autoclaved at 15 lbs/in^2 (121^{0}C) for 15-20 minutes. Test tubes were placed over racks that tilt the test tubes during cooling and gave slanted surface to the agar medium.

Inoculation:

All experimental manipulation was carried out under aseptic conditions in laminar airflow fitted with a bactericidal UV tube (15W, peak emission 2537Å). The floor of chamber was thoroughly scrubbed with cotton dipped in alcohol. The surface of all the vessels, other accessories such as spatula, forceps, needles, and scalped etc., Bunsen burner, matchbox, tube containing absolute alcohol etc. were also cleaned with spirit. Fresh tissue to be inoculated was kept in a Petri plate covered with black paper in order to protect it from harmful ultraviolet rays. Alcohol was sprayed in the chamber with sprayer. 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 inoculation on respective media. Leaf explants, nodal segments and shoot apices were taken from field grown healthy mother plant. These were placed in a bottle 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 rinsed with liquid detergent (1% v/v) for another 15 minutes and then washed properly with tap water to remove the detergent. They were treated with bavistin (0.1% w/v) for 5 to 10 minutes to remove the fungal contamination followed by washing with distilled water. Explants were then surface sterilized with 0.1%(w/v) aqueous solution of $HgCl_2$ for 2-3 minutes for leaf explants and shoot apices and 5-6 minutes for nodal segments followed by 4-5 rinses in sterilized double distilled H_2O in inoculation chamber.

Cultural conditions:

All the cultures were maintained in an air-conditioned room at temperature of 25 ± 4^0C. The source of illumination consisted of 4 feet wide fluorescent tubes (40 W) and incandescent bulb (25 W). The intensity of illumination was 50µm $m^{-2}s^{-1}$ lux at the level of cultures and 12 hours light regime was followed by 12 hours of darkness.

Observations and Results

Young leaves, nodal segments and shoot apices were excised from field grown 4-years-old plant of *Tylophora indica* and were used for *de novo* induction of adventitious shoots and forced axillary branching. The explants (4-5mm) were cultured on MS medium supplemented with different growth regulators used either singly or in combination with each other. Twenty replicates were used for each treatment and cultures were sub cultured after every 7-8 weeks.

De novo adventitious shoot induction from leaf explants

Leaf segments (4-5 mm in size) were cultured on variously supplemented MS medium for *de novo* adventitious shoot formation directly from the explant. Out of all the growth regulators tried, Murashige and Skoog's (MS) medium supplemented with 6-benzyl adenine (8.8μM) either alone or in combination with adenine sulphate (1.35μM) produced greatest number of shoots/explant.

Nodular meristemoids differentiated from the cut ends, abaxial and adaxial surface of leaf lamina on 8.8μM BAP after 8 days (figure. 3) and within 3 weeks the meristemoids covered the entire surface of the leaf (figure. 4). Eventually these meristemoids developed into green leafy shoot in about 80% of cultures. Initially fewer shoots were formed from these meristemoids (figure.5) but in due course, these multiplied further forming many shoots (figure. 6). Murashige and Skoog's medium supplemented with BAP (8.8μM) and adenine sulphate (1.35μM) produced the greatest number of shoot buds directly from the leaf explants. Nodular meristemoids differentiated from the entire cut surface of leaf lamina after 8-10 days of culturing and within 3 weeks entire surface was covered with these meristemoids. Eventually these meristemoids grew into leafy shoot after 5 weeks in 90% of cultures (Figure. 7). The shoots elongated and developed many leaves (figure. 8). Repeated sub culturing accelerated the formation of shoots in large numbers (45-50 per culture) without any decline in proliferation (Figure. 9).

Nodal explant culture

Fresh nodal explants were collected from healthy, field grown mature plant of *Tylophora indica.* 5mm long single node , each holding one dormant lateral bud was excised and surface sterilized with 0.1% mercuric chloride. Damaged intermodal tissue on the both sides

of sterilized segments was cut off. Segments 3-4 mm in size was then cultured on MS medium supplemented with various growth regulators. The axillary shoot proliferation from the cultured explants was remarkably influenced by type and concentration of the growth regulator used.

Multiple shoot formation occurred on MS medium supplemented with different concentrations of BAP (4.4-17.6μM). However, the number of shoot formed varied with the concentration of BAP. Only 3-4 shoot were formed on lower concentration of BAP (4.4μM). Highest number of shoot per culture was obtained on MS medium supplemented with 17.6μM of BAP, where initial bud break occurred after 10-12 days of inoculation (fig.10) leading to the formation of 8-10 shoots from axillary position after 3 weeks (fig.11). The shoots grew continually there after forming nearly 20-25 shoots from single nodal segments after 6 weeks (fig.12).

MS medium supplemented wit BAP (22 μM) in conjunction with NAA (3.6) and L- Ascorbic acid (8.4μM) gave the best proliferation rate. On this medium bud break occurred after 8-10 days of culturing leading to the formation of 5-6 shoots from the axillary position after 3 weeks. At the same time, lower cut ends of the explant formed a cluster of meristemoids (fig.13), which developed into shoots later on (fig. 14). On frequent sub culturing there was a mark increased in the number of meristemoids and the shoots formed (fig.15) and nearly 40-45 shoots were produced after 7-8 weeks of inoculation. Fig.16 depicts numerous shoots formed from the nodal explant after 8 weeks of culturing.

Response of various growth regulators on shoot proliferation from nodal segment is

depicted in table 1.

Table 1

Hormone concentration	Type of explant	No. of shoots per explant
BAP (1ppm)	Nodal	4
BAP (2ppm)	Nodal	26-30
BAP (4ppm)	Nodal	30-34
BAP(5ppm)+ NAA (0.5ppm)	Nodal	20-25
BAP(5ppm)+NAA(0.5ppm)+ Ascorbic acid (100mg/l)	Nodal	45-50

Shoot apex culture

Young healthy shoot apices were collected from field grown mature plant of *Tylophora indica* and cultured on MS medium with different concentration of BAP either alone or in combination with other growth regulators. Best shoot proliferation from the shoot apex occurred on MS supplemented with BAP + NAA + ascorbic acid where initial bud break occurred after 10-12 days (fig. 17). About 75% of the cultures showed meristemoids at the cut ends which developed into green leafy shoots after 3-4 weeks (fig. 18). On frequent sub culturing there was a marked increase in the number of meristemoids and the shoots formed. (Figs. 19 & 20).

Rooting of microshoots

Regenerated microshoots were carefully rescued from the flasks and were inoculated upright in the medium with or without growth hormones. Shoots were inoculated on half strength and full strength BMS medium and MS medium supplemented with different auxins like IBA, IAA and NAA for root initiation. Among the various auxins tested, IBA (9.8µM) showed the good results where the roots initiated after 15 days. Best root initiation, however, occurred on half strength MS medium where healthy roots emerged in 90% of the cultures. The roots were long and white having root hairs. (fig. 21).

Acclimatization and transfer of plantlets to the soil

The rooted plantlets were successively transferred to field conditions through successive hardening stages. The rooted plantlets were safely removed from the culture tubes keeping the roots intact by using forceps and with extreme care to avoid any mechanical injury to the plantlets. Roots were thoroughly washed under tap water to remove traces of agar sticking to them. Firstly, rooted plantlets were acclimatized on moist cotton for 10-12 days (fig. 22) followed by their transfer to plastic cups containing sterile vermicutile (a mixture of soil & vermicompost 1:1), and covered with plastic bags having holes and kept under the culture room conditions for 15 days (fig. 23). The plants were thoroughly watered. The plants with newly formed leaves were shifted to poly bags (fig. 24) and kept in growth room for another 2 weeks. Then plants were shifted to green house and attempts are underway to establish these plantlets in the natural environment (fig. 25).

De novo adventitious shoot formation from leaf explant on BAP Supplemented medium

Figure.3 Nodular meristemoids initiated from leaf explants on MS + BAP(8.8µM) after 8 days of culturing.

Figure.4 Nodular meristemoids covering the entire surface of leaf lamina on MS + BAP (8.8µM) after 21 days of culturing.

Figure.5 Sprouting of shoots from meristemoids after 4 weeks on MS + BAP (8.8µM).

Figure.6 Numerous shoots developing from meristemoids after 6 weeks.

Fig. 3

Fig. 4

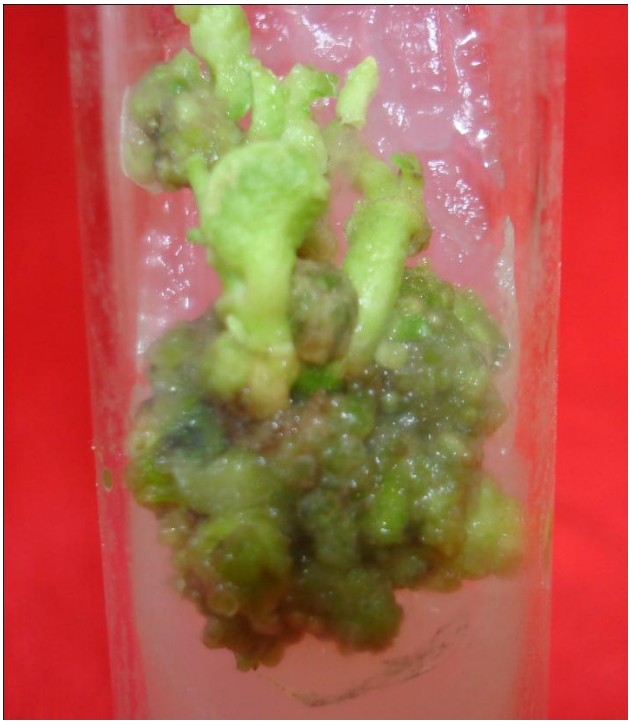


Fig.5

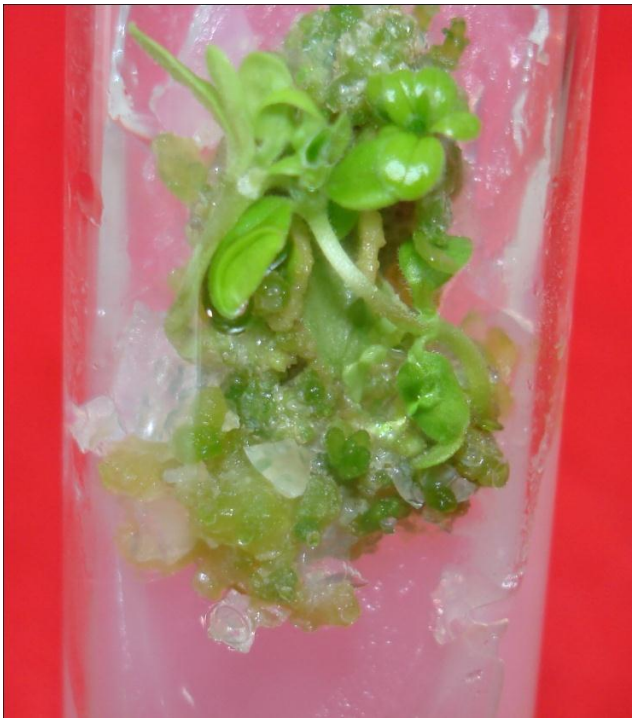


Fig.6

De novo adventitious shoot formation from leaf explant on BAP + Adenine sulphate supplemented medium.

Figure.7 Emgergence of numerous shoots sprouting from nodular meristemoids on MS + BAP (8.8μM) + Adenine sulphate (1.35μM).

Figure.8 Cluster of adventitious shoots formed after 3 weeks on the same medium.

Figure.9 8-week-old culture showing the formation of numerous shoots.

Fig. 7

Fig. 8

Fig. 9

Nodal explant culture

Figure.10 Initial bud break of nodal explant on MS + BAP (8.8μM) after 10-12 days of culturing.

Figure.11 Initiation of multiple shoots from nodal segment after 3 weeks of culturing on 8.8μM BAP.

Figure.12 6-week-old culture showing development of multiple shoots on the same medium medium.

Fig. 10

Fig. 11

Fig. 12

Nodal explant culture

Figure.13 Initial bud break and formation of meristemoids from cut ends of nodal segment after 3 weeks of culturing on MS + BAP (22μM) + NAA (3.6 μM) + L-ascorbic acid.

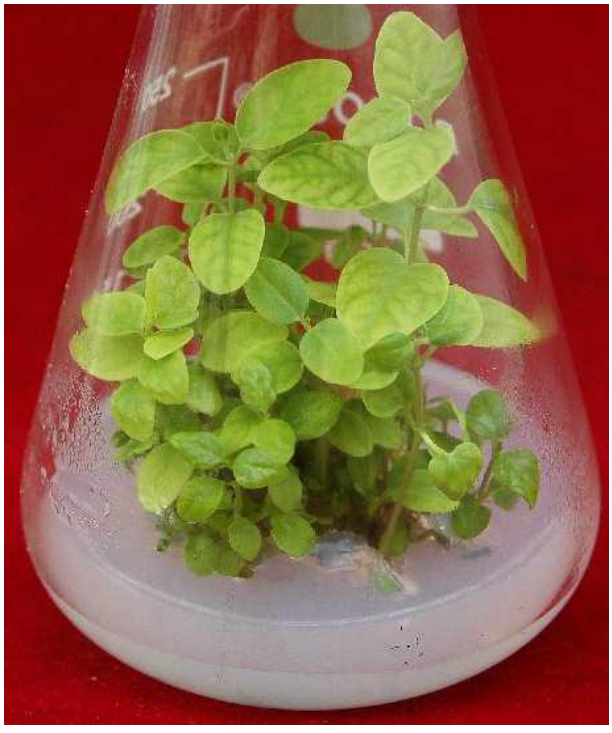
Figure.14 Meristemoids developed into shoots after 4 weeks of culturing.

Figure.15 Increase in number of shoots formed after 5-6 weeks of culturing.

Figure.16 Numerous shoots formed after 8 weeks of inoculation.

Fig. 13

Fig. 14



Shoot apex culture

Figure.17 Initial bud breaks from shoot apex after 10-12 days of culturing on MS + BAP (22µM) + NAA (3.6µM) + L-ascorbic acid (8.4µM).

Figure.18 Meristemoids formed at the cut end of shoot apex developing into leafy shoots after 3-4 weeks.

Figure.19 & 20 Numerous shoots formed from meristemoids after 4 weeks of culturing.

Fig. 17

Fig. 18

Fig. 19

Fig. 20

Rooting and Acclimatization

Figure. 21 Rooting from basal end of regenerated shoot on half strength BMS medium after 15 days of planting.

Figure. 22 The rooted plantlet acclimatization on moist cotton.

Figure. 23 Plantlet transferred to plastic pot containing soil and vermicompost in the ratio of 1:1.

Fig. 24 Plant with newly formed leaves transferred to plastic bag.

Fig. 21

Fig. 22

Fig. 23

Fig. 24

Fig. 25 Acclimatized plants in green house

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.

Tylophora is a threatened medicinal plant having multifarious uses. Due to the lack of the organized cultivation and over exploitation, the wild population of this plant is declining very fast. It is now listed as one of the plant species in India vulnerable to extinction. The commercial plantation of *Tylophora indica* has not been attempted and only wild populations are exploited for secondary metabolite extraction. 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). Therefore, it is necessary to develop an efficient protocol foe large-scale multiplication of this valuble plant species.

De novo adventitious shoot formation directly from leaf explants:

In the present study, *de novo* adventitious shoot formation occurred from the leaf segments on MS supplemented with different concentrations of BAP either alone or in combination with adenine sulphate. Best results, however, were observed on 8.8µM BAP and 1.35µM adenine sulphate where maximum number of shoots (55-60/culture) was formed. Addition of adenine sulphate to the culture medium can stimulate cell growth, greatly enhance shoot proliferation as it has a base structure similar to that of the cytokinin and hence show cytokinin like activity. Subsequent sub culturing further accelerated the formation of shoots in large number without any decline in their proliferation. Periodic sub culturing was essential as otherwise the growth potential was affected which may be due to the accumulation of waste metabolic products in the medium and /or exhaustion of chemicals.

A wide range of cytokinins has been employed for shoot formation and a wider survey suggest that BA is the most reliable and effective cytokinin. The caulogenic effect of BA as described in the present study is in consonance with other reports as well. Bera and Roy, 1993 reported multiple shoot formation directly from leaf explants of *Tylophora indica* on MS medium supplemented with 22 µM BA with 0.65 µM adenine sulphate. Chaudhari et al. (2004) reported the formation of organogenic nodular meristemoids from root explants on MS

medium supplemented with BAP (10.77-26.8μM), which subsequently developed into shoot bud in 42% of the cultures. Likewise Kaur *et al.,* 2011 a,b,c demonstrated BAP with or without adenine sulphate to be most effective in inducing *de novo* adventitious shoots from almost all the vegetative parts including leaf, stem and root explants of *Tylophora indica*.

Multiple shoot proliferation from nodal segments and shoot apices

Micropropagation through axillary and apical shoot proliferation is the most reliable technique for mass propagation since it ensures genetic stability of clones. In present investigation, axillary shoots were induced from nodal segments and shoot apices on MS medium supplemented with different concentrations of BAP alone or in combination with low concentration of NAA and L-ascorbic acid. From a single nodal segment, 20-25 shoots originated on MS medium supplemented with 17.6 μM of BAP after 6 weeks. Initially only 4-5 shoot buds arose per explant after 10-12 days but the number gradually increased up to 20-25 after 4-5 weeks. Best results were, however, occurred on MS medium supplemented with 22μM BAP, 3.67μM NAA and 8.4 μM ascorbic acid where 40-45 shoots could be regenerated from a single nodal segment. Initially 8-10 shoots arose from nodal position but the number gradually increased up to 45-50 after 8 weeks of culturing. Multiple shoot proliferation from shoot apex was also effective on BAP (4.4-22μM) either alone or in combination with NAA (3.6μM) + L-ascorbic acid. Data under discussion is well supported by observations made by Sharma and Chandel, 1992 and Faisal *et al*., 2007 who also employed similar set of media combinations for achieving plant regeneration via enhanced axillary shoot proliferation from nodal segments of *T. indica*.

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 BAP, 2-ip and Zeatin have been employed in shoot proliferation (Bhojwani & Razdan 1983). However, a wider survey of literature suggests that BAP is the most reliable and effective cytokinin for shoot proliferation. The effectiveness of cytokinin especially BAP in promoting axillary shoot proliferation in many medicinal plants is well documented e.g. *Ziziphus* (Sudershan *et al*., 2001), *Rosa damascene* (Pati *et al*., 2004), *Picrorhiza kurroa* (Chandra *et al*., 2006), *Stevia rebaudiana* (Debnath 2008),*Beloperone plumbaginifolia* (Shameer *et al*., 2009) and *Boscia senegalensis* (Khalafalla *et al*., 2011).

Rooting of Microshoots and Acclimatization of *in vitro* plants:

Induction of root at the base of *in vitro* regenerated shoots is an essential and indispensable step to establish tissue culture derived plantlets to the soil. The shoots, thus, formed *in vitro* were excised and transferred on half strength and full strength BMS medium and MS medium supplemented with different auxins like IBA, IAA and NAA for root initiation. Among the various auxins tested, IBA (9.8µM) showed good results where the roots initiated after 15 days of culture. Best root initiation, however, occurred on half strength MS medium where healthy roots emerged in 90% of the cultures. Thomas and Philip (2005) and Faisal and Anis (2005) have reported IBA to be optimal for rooting in the regenerated shoots of *Tylophora indica.* Earlier, Bera and Roy (1992) have reported best rooting from the *in vitro* grown shoots of *Tylophora indica* on IAA supplemented half strength MS medium.

The ultimate success of any micropropagation protocol depends upon the successful transfer and establishment of these plants in the field conditions. Plants produced under *in vitro* conditions under controlled high humidity, diffused light and constant temperature need to be acclimatized because transferring of these plants from *in vitro* to *ex vitro* conditions is the most traumatic experience for them. It is therefore necessary to transfer the plants to field through various hardening stages to increase the survival percentage

In vitro raised plants of *Tylophora* were carefully rescued from the vessels and were initially transferred to the culture bottles containing moist cotton covered with perforated plastic covers to maintain higher relative humidity and were kept for a period of 15 days under growth room conditions. In this period, the plants developed an efficient root system, built up new leaves and became photosynthetically active. Plantlets were then transferred to potting mixture of soil: vermicompost and shifted to green house with 90% survival rate.

It is concluded that the present study demonstrates the establishment of an efficient and reproducible protocol for the production of large number of clones for commercial production of *Tylophora indica*

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