"*In-vitro* Micropropagation of *Adhatoda vasica* Nees. (Basok) for Commercial Exploitation in Bangladesh"

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Examination Roll No: MS-080709

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LIFE SCIENCE SCHOOL, KHULNA UNIVERSITY

KHULNA-9208, BANGLADESH

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KHULNA-9208, BANGLADESH

TITLE

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DECLARATION

This thesis report is the original research work of the author, except as otherwise stated. The material presented has not submitted either in whole or in part for a degree for this or any other university. The findings of the research has not been/will not be published anywhere without the written concurrence of the concerned supervisor. Legal action will be taken in any case of infringement regarding the declaration.

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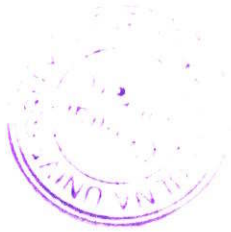

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The Author

ABSTRACT

To enhance the production of the major phytochemical active compounds of *Adhatoda vasica* at large scale, wide cultivation and their conservation as a local valuable medicinal plant attempts were made to establish a protocol *in vitro* micropropagation for large scale production of *Adhatoda vasica.* Nodal segments were used as explants for regeneration of multiple shoot and juvenile leaves were used as explants for callus induction. Among the different growth regulator formulations MS medium supplemented with BAP (10.0 mg/l) was found best for multiple shoot formation, in which 93.33% explants produced multiple shoots, 7 days required for initiation of shooting, the nodal explants remained green and viable for further subculture. After 2 months, maximum number of multiple shoots 10.6 ± 1.82, highest length of plantlet 5.2 ± 2.20 em and maximum number of leaves 15.6± 1.80 were recorded. Maximum 100% callus formation was observed on MS medium supplemented with IAA (0.05 mg/l) + NAA (0.05 mg/l) + BAP (1.0 rng/l). 30 explants were inoculated for callus induction; callus initiation started from 14 days, 30 explants showed callus formation and gave soft, green coloured, fast growing callus. Media containing IBA (1.0 mg/l) with GA_3 (1.0 mg/I) also showed better response, 30 explants were inoculated; callus initiation started from 14 days, 28 explants showed callus formation, 93.33% of responsive explants and callus gives light green colour. Best callus mediated shooting found from MS + 1 0.0 mg/l BAP medium, 10 explants were transferred, 80% explants showed regeneration, 10 days required for initiation of shooting, mean number of multiple shoot 4.0 ± 1.70, mean length of plant let 3.0 ± 1.56 cm and mean number of leaves were 11.0 ± 1.45. Rooting response of *in vitro* raised shoots from nodal explants on MS + IBA (2.5 mg/l) + IAA (0.5 mg/l) + NAA (0.5 mg/l) performed best; root formation of explants was 80%, 12 days required for initiation of rooting from shoot regenerated from nodal explants and, root formation of explants was 60%, 13 days required for initiation of rooting from callus mediated shoot. Well rooted plantlets were transferred to plastic pots containing soil for hardening. All acclimatized plantlets were then transferred to garden soil. The ultimate survival rate of the transferred plantlets to soil was about 78%.

ABBREVIATIONS

% : Percent

°C: Degree Celsius

µl: Micro litre

2,4-D : 2,4-dichlorophenoxy acetic acid

BAP : 6-Benzyl amino purine

cm: Centimeter

DW : Distilled water

e.g. : Example

EDTA: Ethylene diamine tetraacetic acid

et al. : et alia = and others

etc. : et cetera = and the others

Fig. : Figure

GA3: Gibberelic acid

gm : Gram

HCI : Hydrochloric acid

i.e. : Id est = that is

IAA : Indole-3-acetic acid

IBA: Indole-3-butyric acid

Kn : Kinetin (6-ferfuryl amino purine)

L. : Linnaeus

lbs : Pounds(s)

M : Molar

mg : Miligram

mg/l : Miligram per liter

Min : Minute(s)

ml : Mililitre

MS : Murashige and Skoog (1962) medium

N : Normal Solution

NAA : α-napthalene acetic acid

NaOH : Sodium hydroxide

Nees. : Christian Gottfried Daniel Nees von Esenbeck

no. : Number

pH : Negative logarithm of hydrogen ion $[H^+]$ concentration

SE: Standard Error

sp. : Species

Var. : Variety

viz. : Videlicet = Namely

w/v : Weight by volume

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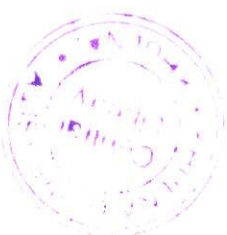


Chapter one

Introduction

1. INTRODUCTION

Plants have played a critical role in maintaining human health and civilizing the quality of human life for thousands of years. The world health organization (WHO) has estimated that 80% of the earth's inhabitant relied on traditional medicine for their primary health care needs and most of these therapies involved the use of plant extract or their active compounds (Bruneton, 1995). The herbal products today symbolize safety in contrast to the synthetics that are regarded as unsafe to human and environment. Although herbs had been priced for their medicinal, flavouring and aromatic qualities for centuries, the synthetic products of the modern age surpassed their importance, for a while. However, the blind dependence on synthetics is over and people are returning to the naturals with hope of safety and security.

Adhatoda vasica is a well known medicinal plant in Bangladesh, belonging to the family Acanthaceae is an evergreen woody shrub widespread throughout the tropical regions of Southeast Asia (Chakrabarty and Brantner, 2001), including Bangladesh. *Adhatoda vasica* is a highly reputed plant in Ayurvedic and Unani medicine. The plants are important natural sources of medicines and pharmaceutical products (Kumar, 2004; Patwardhan et al., 2004). *Adhatoda* leaves have been used extensively in Ayurvedic medicine primarily for respiratory disorders. It is mainly antispasmodic, fever reducer, anti-inflammatory, anti bleeding, branchodilator, antidiabetic, antihelminthic, disinfectant, anti-jaundice, antiseptic, oxitocic and expectorant and has many other medicinal applications (P.K. Patel et al., 1984 and Chakrabarty and Brantner, 2001).

The term "Medicinal" originated from Latin "Medicinalis". A large group of plants used in medicine or veterinary practice for therapeutic or prophylactic purposes. The therapeutic properties of medicinal plants are conditioned by the presence of active substances such as alkaloids, flavonoids, glycosides, vitamins, tannins and coumarine compounds in their organs, which physiologically affect the bodies of humans and animals or which are biologically active in relation to the causative agents of various diseases. A special group of medicinal plants are antibiotics. Medicinal plants are

plants having healing qualities and pertaining to a medicine or to healing. (http://encyclopedia2.thefreedictionary.com/Medicinal+Plants)

Plants have played a critical role in maintaining human health and civilizing the quality of human life for thousands of years. The world health organization (WHO) has estimated that 80% of the earth's inhabitant relied on traditional medicine for their primary health care needs and most of these therapies involved the use of plant extract or their active compounds (Bruneton, 1995). The herbal products today symbolise safety in contrast to the synthetics that are regarded as unsafe to human and environment. Although herbs had been priced for their medicinal, flavouring and aromatic qualities for centuries, the synthetic products of the modern age surpassed their importance, for a while. However, the blind dependence on synthetics is over and people are returning to the naturals with hope of safety and security.

1.1. Plant Description:

1.1.1. General description: *Adhatoda vasica* is a small evergreen sub-herbacious bush, of the acanthaceae family, with broad, lanceolate leaves measuring 10 to 16 centimeters in length and 5 centimeters wide. They are oppositely arranged, smooth-edged, and borne on short petioles. They become greenish-brown when dried and have a bitter taste. They have a smell similar to strong tea. The wood of the stem is soft, and makes a great charcoal for gunpowder. The inflorescence is dense, short pedunculate, bractate and spike terminal. The flower has large, attractive, white petals, streaked with purple on the lower lip. The fruit is a small capsule with 4 seeds. The stomata in the plant are elongated and oval in shape.

1.1.2. Macroscopic description: Leaves, 10-30 cm long and 3-10 cm broad, lanceolate to ovate-lanceolate, slightly acuminate, base tapering, petiolate; petioles 2-8 cm long, exstipulite, glabrescent, 8-10 pairs of lateral vein bearing few hairs; dried leaves dull brown above, light greyish brown below; characteristic odour; bitter taste.

1.1.3. Microscopic description: Transverse section of leaf shows, dorsiventral surface with 2 layers of palisade cells; in surface with 2 layers of palisade cells; in surface view, epidermal cells sinuous with anomocytic stomata on both surfaces, more numerous on the lower; clothing trichomes few, 1-3 rarely upto 5 celled, thin-

walled, uniseriate, upto 500 μ and glandular trichomes with unicellular stalk and 4 celled head measuring, 25-36 μ in diameter in surface view; cystoliths in mesophyll 1 years, elongated and cigar shaped; acicular and prismatic forms of calcium oxalate crystals present in mesophyll; palisade ratio, 5-6, 5-8.5; stomatal index, 10.8-14.2-18.1 for lower surface.

1.2. Scientific Classification of *Adhatoda vasica*:

Bengali name	: Basok.
Other common names	: Malabar nut, adulsa, arusha, vasaka, adulsa arusa, adathodai, bakash, adathoda, adalodakam, adusoge, addasaramu, lion's muzzle, atallion's tooth.
Botanical name	: *Adhatoda vasica, Justicia adhatoda.*
Kingdom	: Plantae
Division	: Magnoliophyta
Class	: Magnoliopsida
Subclass	: Asteridae
Order	: Lamiales
Family	: Acanthaceae
Genus	: *Adhatoda*
Species	: *A. vasica* Nees.

1.3. History and Habitat:

Adhatoda leaves have been used extensively in Ayurvedic Medicine for over 2000 years primarily for respiratory disorders. *Adhatoda vasica* grows commonly in the open plains especially in the lower Himalayans, up to a range of 1000 meters above sea level. It grows in India, Sri Lanka, Burma and Malaysia. This plant is also cultivated in other tropical areas. It will grow well in low moisture areas and dry soils. In Bangladesh it is cultivated in large gardens in Habiganj, Jessore, Maniganj and Satkhira districts. (Sukhdev S. H., Dev D. R., Karan V., 2006.)

1.4. Cytological Studies of *Adhatoda vasica*:

Chromosome number: 2n = 34.

Chromosomal association bivalent (range / average ± S.E.) ring: 6-8/6.4±0.466.

Chromosomal association bivalent (range / average ± S.E.) rod: 9-10/8.0±0.986.

Univalent (range / average ± S.E.): 2-4/3.2±0.430.

Chiasma frequency / chromosome (range / average ± S.E.): 20.23/21.8±0.609.

(Seema S. *et al.*, 2010).

1.5. Plant Constituents of *Adhatoda vasica*:

Leaves:

- Quinazoline alkaloids
- Vasicine- 45-95% (the mucolytic drug bromhexine was developed from this alkaloid).
- N-oxides of vasicine
- Vasicinone
- Vasicinol
- Adhatodine
- Adhatonine
- Adhvasinone
- Anisotine
- Hydroxypeganine
- Deoxyvasicine
- Oxyvasicinine
- Maiontone
- Essential oil.

The leaf extract, is considered safe and the oil has low toxicity.

Flowers:

- b-sitosterol-D-glucoside
- Kaempferol
- Glycosides of kaempferoland
- Triterpenes (α-amyrin)
- Apigenin
- Astragalin
- Vitexin
- Queretin
- Alkanes

Roots:

- Vasicinolone
- Vasicol

- Peganine
- Hydroxy oxychalcone
- Glucosyl oxychalcone.

Other:
- Odorous volatile principle
- Organic adhatodic acid.

1.6. Phytochemistry:

The chemical compounds found in *Adhatoda vasica* plant includes essential oils, fats, resins, sugar, gum, amino acids, proteins and vitamins 'C' etc (Bhat et al., 1978). The phytochemical analysis show that phenols, tannins, alkaloids, anthraquinone, saponins, flavonoids and reducing sugars were found in the leaves of *Adhatoda vasica* (Pathak, 1970). But the pharmacologically most studied chemical component in *Adhatoda vasica* is a bitter quinazoline alkaloid, vasicine (1, 2, 3, 9-tetrahydropyrrole [2, 1-b] quinozolin-3-ol, $C_{11}H_{12}N_2O$) which is present in the leaves, roots and flowers. It can be synthesized by addition of 2-aminobenzylamine to the vicinyl vicinal trycarbonyl reagent which leads to the short synthesis of Vasicine (Wasserman and Kuo, 1991). Besides vasicine, the leaves contain several alkaloids (Vasicinone, Vasicinol, Adhatodine, Adhatonine, Adhvasinone, Anisotine and Hydroxypeganine), betaine, steroids and alkanes (Lahiri and Prahdan, 1964; Chowdhury and Bhattacharyya, 1987). Vasicine is metabolized to vasicinone and analysis of *Adhatoda vasica* leaf extract showed that it contained 0.85% vasicine and 0.027% vasicinone.

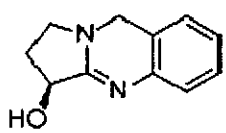


Figure-1: Chemical structure of Vasicine ($C_{11}H_{12}N_2O$)

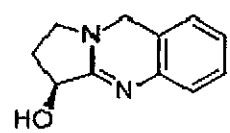


Figure-2: Chemical structure of Vasicinone ($C_{11}H_{10}N_2O_2$)

The absolute stereochemistry of (-)-Vasicine and (-) -Vasicinone have been shown the 3S configuration on the basis of X-ray analysis of the alkaloid hydrobromides. Similarly, Vasicinol and Vascinolone which have been interrelated should also have

the 3S configuration (Joshi et al., 1996). The novel alkaloid isolated leaves and characterized as 1, 2, 3, 9-tetrahydro- 5-methoxypyrrol [2, 1-b] quinazolin-3-ol (Chowdhury and Bhattacharyya, 1987). The roots also contain alkaloids (vasicinal, vasicinolone, vasicinone and adhatonine), a steroid (daucosterol), carbohydrates and alkanes. In the flowers triterpenes (α-amyrin), flavonoids (Apigenin, Astragalin, Kaempferol, Quercetin, Vitexin) and alkanes have been found (Haq et al., 1967).

1.7. Pharmacology of *Adhatoda vasica*:

The major phytochemical active compounds like vasicine and vasicinone which are isolated from water and alcoholic extracts of *Adhatoda vasica* exert effective pharmacological actions. Such as-

- Abortifacient [an agent that induces or causes premature expulsion of a fetus (abortion)]
- Anti-asthmatic [an agent to relieve asthma]
- Antispasmodic [an agent which relieves or eases muscular spasms, cramps or convulsions]
- Antitussive [an agent that relieves coughing]
- Bronchodilator [an agent to dilate the bronchials]
- Expectorant [an agent that promotes the discharge of mucous and secretions from the respiratory passages]
- Febrifuge [an agent that reduces or eliminates fevers]
- Mucolytic [an agent that thins and breaks down mucus]
- Oxytocic [an agent that stimulates contraction of the uterine muscle, facilitating or speeding up childbirth]
- Uterotonic [an agent that tones, strengthens and invigorates the uterus or the entire organism giving a feeling of well-being]
- Anti-inflammatory [an agent having the property that reduces inflammation (part of the complex biological response of vascular tissues to harmful stimuli, such as pathogens, damaged cells, or irritants)]
- Cardioprotective [an agent that protects heart & effective in stimulating cardiac muscles].
- Antibacterial [an agent kills or slows down the growth of bacteria].

Essential oils, which are also known to contain ketone, terpene, and phenolic ether, have antitumor, antioxidant, antiaging, antimutation and sedative effects, plus the high phenolic derivative content of essential oils contributes to their antimicrobial properties (Bandini *et al.* 1981 & Wealth of India).

1.8. Medicinal uses of *Adhatoda vasica*:

Plant Parts Used:

The leaves, roots, flowers and stem bark of *Adhatoda vasica* are used in medicinal applications. Mainly, leaf extracts are widely used for medicinal purpose.

Adhatoda vasica is commonly used for:

- Blood Conditions.
- Bleeding due to idiopathic (unknown) thrombocytopenic (blood does not have enough platelets) purpura (excessive bruising)

Cardiovascular Conditions

- Moderate hypotensive activity (lowers blood pressure)
- Pulmonary diseases

Female Conditions

- Assists uterine involution (rolling or turning inward)
- Menorrhagia (excessive menstrual bleeding)
- Postpartum (after childbirth) hemorrhage
- Uterine stimulant activity

Gastrointestinal conditions

- Dyspepsia
- Local bleeding due to peptic ulcer and/or piles (hemorrhoids)

Respiratory Tract Conditions

- Acute and chronic bronchitis often combined with the herbs Licorice and Marshmallow for soothing the respiratory tract
- Allergic asthma, often combined with the herbs Albizia, Baical Skullcap, Eyebright and Tylophora
- Antihistamine effects (the leaves may be dried and smoked to relieve asthma)
- Broncho-dilation effects
- Emphysema

- Obstructive airway disease (chronic) often combined with the herbs Polygala, Grindelia, Euphorbia and Elecampane
- Phthisis (wasting of the body as in tuberculosis)
- Relieves cough and breathlessness.

Other Conditions

- Bleeding gums (applied locally)
- Diphtheria
- Gives relief in pyorrhoea, a gum disease (applied locally)
- Intermittent fever
- Tuberculosis (all strains of inhibited by the essential oil)
- Typhus fever

Dosage:

Recommended dosage is as follows:

- 0.5-1.5g/day dried root
- 1-3mL/day 1:2 fluid extract

Higher doses may be needed for the oxytocic effect during childbirth [to stimulate contraction of the uterine muscle, facilitating or speeding up childbirth]

1.9. Contraindications of *Adhatoda vasica*:

Adhatoda vasica was traditionally used by midwives at the time of delivery because of its uterotnoic activity. *Adhatoda vsica* is reported to have oxytocic (stimulates contractions of the uterus) and abortifacient (substance that induces abortion) effects. Due to its anti-implantation activity, *Adhatoda* should not be used while pregnant. Large doses cause diarrhoea, irritation of the alimentary canal and vomiting. (http://www.globalherbalsupplies.com)

1.10. Reasons for the Reduction of Natural Resources of Medicinal plants:

1. Lack of knowledge about the value of the forest, resulting in the degradation of this resource base.

2. Natural calamities, like landslides, uplifting or sinking of land, flood, glaciation, droughts, etc.

3. Disease, insect attack, absence of pollination and inadequate reproductive mechanisms.
4. Haphazard and commercial exploitation of roots, barks, rhizomes, corms, seeds, flowers and fruits.
5. Deliberate setting of fires in the forest.

1.11. Conservation and Cultivation of Medicinal Plants:

Most of medicinal plants, even today, are collected from wild. The continued commercial exploitation of these plants has resulted in receding the population of many species in their natural habitat. Vacuum is likely to occur in the supply of raw plant materials that are used extensively by the pharmaceutical industry as well as the traditional practitioners. Consequently, cultivation of these plants is urgently needed to ensure their availability to the industry as well as to people associated with traditional system of medicine. If timely steps are not taken for their conservation, cultivation and mass propagation, they may be lost from the natural vegetation forever. *In situ* conservation of these resources alone cannot meet the ever increasing demand of pharmaceutical industry. It is, therefore, inevitable to develop cultural practices and propagate these plants in suitable agroclimatic regions. Commercial cultivation will put a check on the continued exploitation from wild sources and serve as an effective means to conserve the rare floristic wealth and genetic diversity. It is necessary to initiate systematic cultivation of medicinal plants in order to conserve biodiversity and protect endangered species. In the pharmaceutical industry, where the active medicinal principle cannot be synthesized economically, the product must be obtained from the cultivation of plants. Systematic conservation and large scale cultivation of the concerned medicinal plants are thus of great importance (Joy P. P. et al 1998). The increasing demand for the raw materials of medicinal plants, plant tissue culture or in vitro micropropagation is an effective tool for mass propagation of these valuable plants to conserve them and to make them commercially available.

1.12. Plant Tissue Culture:

Plant tissue culture is a collection of techniques used to maintain or grow plant cells, tissues or organs under sterile conditions on a nutrient culture medium of known composition. Plant tissue culture is widely used to produce clones of a plant in a method known as micropropagation. Different techniques in plant tissue culture may

offer certain advantages over traditional methods of propagation, Plant tissue culture is based on the cell doctrine that states a cell is capable of autonomy and is potentially totipotent. In 1902, the German botanist Gottlieb Haberlandt developed the concept of *in vitro* cell culture. Therefore, Haberlandt is considered as the father of plant tissue culture. In 1939, Gautheret cultivated cambial tissues of carrot root, Nobecourt (carrot), and White (tobacco) for prolonged periods of time. In strict sense, these were the first true plant tissue cultures (Chawla, H.S., 2002).

The technique of plant tissue culture occupies a key role in the second green revolution in which gene modification and biotechnology are being used to improve crop yield and quality. Those working with plant cell tissue culture are still concerned with the details of the technique but are now more involved in its application to fundamental aspects of plant cell differentiation and development and to the problems of crop improvement (Hamish A. Collin, S. Edwards., 1998). *In vitro* techniques are important tools for modern plant improvement programs to introduce new traits into selected plants, to multiply elite selections and to develop suitable cultivars in the minimum time (Taji *et al.* 2002). Used in conjunction with classical breeding methods, an efficient *in vitro* shoot proliferation and regeneration system could accelerate cultivar development programs. The ability to regenerate plants is crucial to the successful application of *in vitro* methods (Cao and Hammerschlag 2000).

1.13. Applications of tissue culture:

Micropropagation is widely used in forestry and in floriculture. Micropropagation can also be used to conserve rare or endangered plant species. A plant breeder may use tissue culture to screen cells rather than plants for advantageous characters, e.g. herbicide resistance/tolerance. Large-scale growth of plant cells in liquid culture in bioreactors for production of valuable compounds, like plant-derived secondary metabolites and recombinant proteins used as biopharmaceuticals. As a tissue for transformation, followed by either short-term testing of genetic constructs or regeneration of transgenic plants. Certain techniques such as meristem tip culture can be used to produce clean plant material from virused stock, such as potatoes and many species of soft fruit. Micropropagation using meristem and shoot culture to produce large numbers of identical individuals. Production of identical sterile hybrid species can be obtained through tissue culture.

1.14. Advantages of Micropropagation over Traditional Methods of Propagation:

Plant tissue culture relies on the fact that many plant cells have the ability to regenerate a whole plant (totipotency). Single cells, plant cells without cell walls (protoplasts), pieces of leaves, or (less commonly) roots can often be used to generate a new plant on culture media given the required nutrients and plant hormones. Advantages of tissue culture over traditional methods of propagation are listed as-

- The production of exact copies of plants that produce particularly good flowers, fruits, or have other desirable traits.
- To quickly produce mature plants.
- The production of multiples of plants in the absence of seeds or necessary pollinators to produce seeds.
- The regeneration of whole plants from plant cells that have been genetically modified.
- The production of plants in sterile containers that allows them to be moved with greatly reduced chances of transmitting diseases, pests, and pathogens.
- The production of plants from seeds that otherwise have very low chances of germinating and growing, i.e.: orchids and nepenthes.

To clean particular plants of viral and other infections and to quickly multiply these plants as 'cleaned stock' for horticulture and agriculture.

1.15. Objectives:

1. To establish callus culture, for indirect regeneration of *Adhatoda vasica* (Basok).
2. To produce large amount of callus through callus culture to get some important secondary metabolites.
3. To find out the effect of some growth regulators or supplements on callus culture of *Adhatoda vasica* leaf.
4. To establish protocol for large scale production of *in-vitro* plantlets from nodal and shoot tip explants of mature plants.
5. To explore anti-microbial activity of *Adhatoda vasica.*
6. To produce disease free plantlets of *Adhatoda vasica.*

Chapter two

Materials and Methods

2. MATERIALS AND METHODS

2.1. Materials:

The following materials were used in the investigation:

- Plant materials
- Basic organization and equipment facilities

2.1.1 Plant Materials:

Fresh young leaves and nodal segments from the top of the branch of *Adhatoda vasica* were collected from field grown plants in garden of Tissue Culture Section, Biological Research Division, Bangladesh Council of Scientific & Industrial Research (BCSIR) Laboratories, Dhaka (Fig.-3).

Figure 3: *Adhatoda vasica* plant in the garden of BCSIR

2.1.2 Basic Organization and Equipment Facilities:

Tissue culture work was performed in a clean room of tissue culture lab, BCSIR Dhaka having provision for working space, free of dust and convection current that carry spores of microorganisms. The preparation room was equipped with the following:

- Cabinet and shelf space for safe storage of chemical and dust free storage for clean glass wire.
- Transfer areas for aseptic manipulation.
- Analytical and top loader balances.
- Steam autoclave and microwave oven for sterilizing media, solution,

water, culture vessels and instruments.
• Culture room where cultures were incubated under controlled light and temperature.
• A supply of distilled and double distilled water.
• Various instruments and appliances, such as-
- Laminar air flow cabinet
- Spirit lamp
- pH meter
- Magnetic hot plate stirrer
- Electric balance
- Scientific refrigerator
- Microscope
- Shaker
- Drying oven
- Tweezers, forceps and scalpels
- Labels and marking pens etc.

2.2. Methods:

The experiment was carried out with the following sequential steps-

2.2.1. Preparation of Culture Media:
MS media (Murashige and Skoog, 1962) with different concentrations of plant growth regulators were used for the tissue culture of Adhatoda vasica. The first step of the preparation of the medium was the preparation of the stock solution of different concentrations.
The various constituents of the medium were prepared into stock solutions for ready use during the preparation of the medium. As different constituents were required in different concentrations, separate stock solutions for macronutrients, micronutrients, Fe-EDTA, vitamins, plant growth regulators etc. were prepared.

2.2.2. Preparation of Stock Solution:
Stock solution I (Macronutrients):
List of components of Stock solution I (Macronutrients) according to Murashige and Skoog, 1962.

Chemical Compounds	mg/L	20X(in grams)
1. NH_4NO_3	1650	33.0
2. KNO_3	1900	38.0
3. $CaCl_2.2H_2O$	440	8.8
4. $MgSO_4.7H_2O$	370	7.4
5. KH_2PO_4	170	3.84

This stock solution was made in such way that its strength was 20 times (20X) more than the final strength of the medium in 1.0 litre of distilled water. For this purpose, 20X weight of different salts required for 1.0 litre of medium were weighted accurately. Then these salts were sequentially dissolved in distilled water one after another in a beaker through magnetic stirrer. Then this solution was taken in a volumetric flask and the final volume of the solution was made up to 1.0 litre by addition of distilled water. The stock solution was then filtered and poured into a clean plastic container and labeled. The solution was stored in a refrigerator at 4°C for further use.

Stock solution II (Micronutrients):

List of components of Stock solution II (Micronutrients) according to Murashige and Skoog, 1962.

Chemical Compounds	mg/L	(100X) (in grams)
1. KI	0.83	0.083
2. H_3BO_3	6.2	0.62
3. $MnSO_4.4H_2O$	22.3	2.23
4. $ZnSO_4.7H_2O$	8.6	0.86
5. $Na_2MoO_4.2H_2O$	0.25	0.025
6. $CuSO_4.5H_2O$	0.025	0.0025
7. $CoCl_2.6H_2O$	0.025	0.0025

This part of the stock solution was made with the micronutrients except $FeSO_4.7H_2O$ and Na_2-EDTA.$2H_2O$. It was made 100 times (100X) the final strength of necessary components in 1.0 litre of distilled water as described for the stock solution I. The solution was filtered, labeled and stored in a refrigerator at 4°C for further use.

Stock solution III (Fe-EDTA):

List of components of Stock solution III (EDTA) according to Murashige and Skoog, 1962.

Chemical Compounds	mg/L	(100X) (in grams)
1. $FeSO_4.7H_2O$	27.8	2.78
2. Na_2-EDTA.$2H_2O$	37.3	3.73

The solution was also made 100 times (100X) the final strength of $FeSO_4.7H_2O$ and Na_2-EDTA.$2H_2O$ in 1.0 litre distilled water in a beaker and heated slowly at low temperature until the salts dissolved completely. Finally the solution was filtered, labeled and stored in a refrigerator at 4°C for further use.

Stock solution IV (Vitamins):

List of components of Stock solution IV (Vitamins) according to Murashige and Skoog, 1962.

Chemical Compounds	mg/L	(100X) (in grams)
1. Nicotinic Acid	**0.5**	**0.05**
2. Pyridoxine HCl	**0.5**	**0.05**
3. Thiamine HCl	**0.1**	**0.01**
4. Glycine	**2.0**	**0.20**
5. Myo-inositol	**100**	**10.0**

Stock solution IV was also made 100 times (100X) the final strength of the medium in 1.0 litre of distilled water as described for stock solution I, II, & III. This solution was also filtered, labeled and stored in a refrigerator at 4°C for further use.

2.2.3. Preparation of Plant Growth Regulators (Hormone):

	Growth Regulators	
Cytokinin	6-Benzyl amino purine	BAP
	6-ferfuryl amino purine	Kn
	Napthalene acetic acid	NAA
Auxin	Indole-3-acetic acid	IAA
	Indole-3-butyric acid	IBA
	2,4-dichlorophenoxy acetic acid	2,4-D
Gibberalic acid	Gibberalic acid	GA_3

10 mg (0.01 gm) Hormone will be dissolved in 100 ml distilled water.
Most of the Hormones should be dissolved firstly in 0.1N NaOH. But 2-4-D should be dissolved firstly in 70% Alcohol, and then the total volume will be filled up with distilled water.

2.2.4. Different Steps of Media Preparation:

To prepare 1.0 litre (1000 ml) of MS medium, the following steps were followed:

- From each of the stock solution, the following amount was added in a 1000 ml beaker- a) 50 ml of stock I
  b) 10 ml of stock II
  c) 10 ml of stock III
  d) 10 ml of stock IV
- 30 grams of sucrose were added in this solution for 1000 ml MS medium, distilled water was added and stirred on magnetic stirrer to completely dissolve the sucrose.
- Different concentrations of hormonal supplements were added to the solution either in single or in combinations as required and mixed well.

- Then the whole mixture was made up to 1000 ml with further addition of distilled water.
- The pH of the medium was adjusted at 5.80, with a digital pH meter by the addition of 0.1N NaOH or 0.1N HCl.
- Agar normally 5% but vary for agar product and temperature. 7 grams of agar were added per litre of medium and heated in a microwave oven until the entire agar was dissolved clearly. If required, it was then gently stirred with spatula.
- The hot media was then poured into culture vessels or conical flasks, after this the culture vessels were tightly plugged up with non-absorbent cotton plugs and marked specifically for different medium with a glass marker to indicate specific hormonal combinations.
- Then the culture vessels were autoclaved at 121°C at 15 lbs of pressure for 20 minutes.
- The media were allowed to cool on the shelf in culture room. These were used in the next for micropropagation.

2.2.5. Culture Method:

2.2.5.1. Choice of Explants for callus Induction and Regeneration:

Fresh juvenile leaves and nodal segments from the top of the branch of *Adhatoda vasica* were collected from field grown plants for callus induction and direct regeneration respectively. Young leaves have capacity of rapid cell division, and is suitable for callus induction and nodal segments from the top have proper regeneration capacity.

2.2.5.2. Surface Sterilization of Explants:

The leaves and nodal segments of *Adhatoda vasica* were surface sterilized. Surface sterilization was done by the following steps-

- Fresh leaves and nodal explants were taken in a beaker and kept under running tap water for 5 minutes to reduce microbial and fungal load.
- Leaves and nodal explants were then washed with mild detergent for two times, and washed thoroughly with water to remove any traces of detergent.
- 5-6 drops of Savlon, an antiseptic solution was added with water and the leaves and nodal explants were dipped into it, shaking the beaker for a while, then these were rinsed off with water.
- 1 drop of Tween-20, a popular non-ionic detergent was used to wash the leaves and nodal explants for two times and washed thoroughly with water to remove any traces of Tween-20.
- Then these were washed with distilled water for two times.
- After distilled water washing these leaves and nodal explants were taken into the laminer air flow cabinet and poured into an autoclaved beaker and washed with 70% alcohol for 1 minute then rinsed off with autoclaved distilled water.

- Finally these explants were treated with 0.1% $HgCl_2$ to avoid microbial and fungal growth during micropropagation period. After addition of 0.1% $HgCl_2$ the beaker was shaking continuously for 3 minutes for leaves and 5 minutes for nodal explants, then these leaves were washed very well with autoclaved distilled water for 3 times.

After surface sterilization, the leaves and nodal explants were ready for inoculation on the culture medium.

2.2.5.3. Inoculation and Maintenance:

Surface sterilized leaves were transferred to a sterile petridish. A leaf was excised into small pieces about 1.0 cm long and 1.0 cm width for callus induction with the help of sterilized scalpel and forceps. The excised leaf explants were then inoculated into agar solidified MS medium containing various hormonal supplements. In each conical flask four to five pieces of excised parts were placed.

Surface sterilized nodal segments were also transferred to another petridish and about 2-3 cm length were excised with the help of sterilized scalpel and forceps. The excised nodal segments were then inoculated vertically into test tubes containing shoot regeneration media. In each test tube a single cutting was placed.

These cultures were maintained at a temperature of 25±2°C with a photoperiod 12 hours per day. The photoperiod was maintained by an automatic timer system.

2.2.5.4. Sub-culture

a) Sub-culture of multiplication of shoots from nodal explants and callus:when the regenerated shoots were 4-5 cm in length, they were removed aseptically from the culture vessels and placed on a sterile Petridis and cut into convenient sizes and again transferred into test tube containing the same supplemented media.
b) Sub-culture for increasing mass of callus: When callus were found at a great mass on the surface of the medium, it was needed for increasing the mass of callus. For this instance, the certain callus responding hormone containing media was made; the callus was removed aseptically from culture vessels and placed on a sterile Petridis and transfer to the same hormone supplement media.
c) Sub-culture for induction of shoot from callus: For induction of shoot from callus, the callus was removed aseptically from culture vessels and placed in a sterile Petridis and transfer to a appropriate (which was responsible for shoot induction from callus) hormone supplement media.
d) Sub-culture for root induction from shoots: when the regenerated shoots were 4- 5cm in length with strong inner core, they are rescued aseptically from the culture vessels and placed on a sterilized Petridis. Basal end of the shoot were then each of the shoot was inoculated on freshly prepared medium containing half strength of MS and MS medium with different combination and concentration of hormone supplements for shoot induction.

2.2.6. Collection of Data:

Data were collected after 30 and 60 days of each culture inoculation with the following parameters and the methods:

2.2.6.1. For Shoot Induction from Nodal Explants:

a) Percentage of explants showed regeneration:

The percentage of explants showed regeneration of multiple shoots was calculated using following formula:

$$(\%)\ of\ explants\ showed\ regeneration = \frac{Number\ of\ responsive\ explant}{Total\ number\ of\ explants\ inoculated} \times 100$$

b) Number of shoot per explants:

Number if shoots per explants was counted after 30 days. Mean number of adventitious shoots per explants was calculated using following formula

$$\text{Mean number of shoot} = \frac{Total\ number\ of\ shoot}{Number\ of\ observations}$$

c) Length of shoot or plantlet:

Length of shoot was measured in cm scale for each explant. Average length of shoot was calculated by using the following formula:

$$\text{Mean length of shoot} = \frac{Total\ length\ of\ shoot}{Number\ of\ observations}$$

2.2.6.2. For Callus Induction from Leaves:

a) Percentage of explants induced callus:

The percentage of explants induced to develop callus were calculated using following formula:

$$(\%)\ \text{of callus inducing explants} = \frac{Number\ of culture\ induced\ callus}{Total\ number\ of\ explants\ inoculated} \times 100$$

2.2.6.3. For Root Induction:

a) Percentage of explants induced roots:

Frequency of shoots induced to develop roots was calculated by using the following formula:

$$(\%)\ \text{of shoot induced roots} = \frac{Number\ of\ culture\ induced\ root}{Total\ number\ of\ explants\ inoculated} \times 100$$

2.2.6.4. Statistical Analysis of Data:

Standard error is usually estimated by the sample estimate of the population standard deviation (sample standard deviation) divided by the square root of the sample size (assuming statistical independence of the values in sample):

$$Se = \frac{S}{\sqrt{n}}$$

Where

S is the sample standard deviation (i.e., the sample based estimate of the deviation of the population)
n is the size (number of observations) of the sample.

2.3. Precautions for Tissue Culture:

Adequate precautions should be taken for tissue culture.

1. The surface of inoculation chamber should be cleaned with 70% alcohol.
2. While working inside laminar flow, hand washed with 70% alcohol. Then transfer the material on fresh culture medium.
3. Equipments like, distilled water, Petridish, vials, forceps, scalpel and also the culture media should be previously autoclaved.
4. $HgCl_2$ (Mercuric Chloride) is a carcinogenic agent (cancer-causing), should be handled with very carefully, while treating the explant.
5. Before and after each transferred material forceps and scalpel washed with spirit (100% alcohol) and also bum with the help of spirit lamp.
6. Removal of cotton plug, the neck of test tube has to flame. After transferring explants in the nutrient medium the autoclaved tube should plugged tightly, which then inoculated under controlled condition where they multiply and grow properly.
7. All operations are done near spirit flame.
8. All cultures were maintained under cool white, fluorescent light with a 12 hour photoperiod, the intensity of lights is 3000 lux, at 25± 2°C temperature.

Chapter three

Result

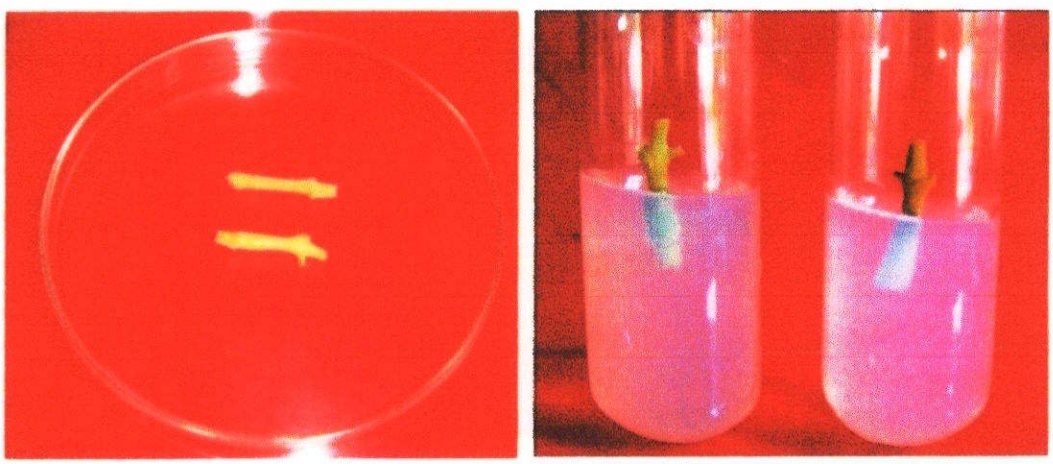
3. RESULTS

An important medicinal plant *Adhatoda vasica* was used in this present investigation for induction of callus, *in vitro* regeneration of multiple shoots from nodal segments and *in vitro* initiation of rooting. This was performed by-

1. Regeneration from nodal explant of *Adhatoda vasica*
2. Callus formation from leaves of *Adhatoda vasica.*

3.1. Regeneration from Nodal Explant of *Adhatoda vasica*:

Regeneration from nodal explant was performed to produce multiple shoots of *Adhatoda vasica*. Nodal explants ranging in size from 1.5 to 2.0 cm long were inoculaed on agar solidified MS media **(Fig.-4)** supplemented with BAP, IAA, NAA, GA_3 and Kn singly or in combinations. 15-20 days after initiation of shooting, explants were subcultured **(Fig.-5).** After 30 and 60 days from inoculation, no. of responsive explant, % of explant showed re-generation, days required for initiation, number of multiple shoot, length of plantlet, size of leaf, number of leaf were recorded. Results of these observations are presented below:



(A) **(B)**

Figure-4: Nodal explants of Adhatoda vasica. (A) Excised nodal segments (B)Explants inoculated on agar solidified MS media supplemented with hormones

3.1.1. Effect of MS medium supplemented with different concentration of BAP on shoot induction from nodal explant:

Different concentrations of BAP (0.5 to 10.0 mg/l) were supplemented with MS media. Results of these observations are presented in **Table-1**. 30 explants were inoculated; shoot initiation started from 7 to 18 days, 10 to 28 explants showed shoot initiation. Mean number of multiple shoot ranges from 1.4 to 5.6. Mean length of plantlet ranges from 0.56 to 5.2 cm. Mean size of leaf is recorded from 0.8 to 1.8 cm and 2 to 15.6 mean number of leaf were found. Best result was found from MS + 10.0 mg/l BAP medium, 28 of responsive explant, 93.33% explant showed regeneration, 7 days required for initiation of shooting, mean number of multiple shoot 5.6, mean length of plantlet 5.2 cm, mean size of leaf is 1.8 cm and mean number of leaf were 15.6. Poor result was found from MS + 0.5 mg/l BAP medium, 10 of responsive explant, 33.33% explant showed regeneration, 18 days required for initiation of shooting, mean number of multiple shoot 1.4, mean length of plantlet 0.56 cm, mean size of leaf is 0.8 cm and mean number of leaf were 2.

3.1.2. Effect of MS medium supplemented with different concentration of BAP and 0.5 mg/l Kn on shoot induction from nodal explant:

Different concentrations of BAP (5.0 to 10.0 mg/l) with 0.5 mg/l Kn were supplemented with MS media. Results of these observations are presented in **Table-2**. 30 explants were inoculated; shoot initiation started from 10 to 15 days, 15 to 21 explants showed shoot initiation. Mean number of multiple shoot ranges from 2 to 4. Mean length of plantlet ranges from 2.0 to 4.6 cm. Mean size of leaf is recorded from 1.0 to 1.5 cm and 6 to 10 mean number of leaf were found. Best result of this combination was found from MS media supplemented with 10.0 mg/l BAP and 0.5 mg/l Kn, 21 of responsive explant, 70% explant showed regeneration, 10 days required for initiation of shooting, mean number of multiple shoot 4, mean length of plantlet 4.6 cm, mean size of leaf is 1.5 cm and mean number of leaf were 10. Poor result of this combination was found from MS media supplemented with 5.0 mg/l BAP and 0.5 mg/l Kn, 15 of responsive explant, 50% explant showed regeneration, 15 days required for initiation of shooting, mean number of multiple shoot 2, mean length of plantlet 2 cm, mean size of leaf is 1.0 cm and mean number of leaf were 6.

3.1.3. Effect of MS medium supplemented with different concentration of BAP and 0.5 mg/l NAA on shoot induction from nodal explant:

Different concentrations of BAP (5.0 to 10.0 mg/l) and 0.5 mg/l NAA were supplemented with MS media. Results of these observations are presented in **Table-3**. 30 explants were inoculated; shoot initiation started from 13 to 18 days, 23 to 27 explants showed shoot initiation. Mean number of multiple shoot ranges from 2 to 4. Mean length of plantlet ranges from 2 to 5 cm. Mean size of leaf was recorded from 1.0 to 1.5 cm and 6 to 14 mean number of leaves were found. Best result of this combination was found from MS media supplemented with 10.0 mg/l BAP and 0.5 mg/l NAA, 27 of responsive explant, 90% explant showed regeneration, 13 days required for initiation of shooting, mean number of multiple shoot 4, mean length of plantlet 5 cm, mean size of leaf was 1.5 cm and mean numbers of leaves were 14. Poor result of this combination was found from MS media supplemented with 5.0 mg/l BAP and 0.5 mg/l NAA, 23 of responsive explant, 76.67% explant showed regeneration, 18 days required for initiation of shooting, mean number of multiple shoot 2, mean length of plantlet 2 cm, mean size of leaf is 1 cm and mean numbers of leaves were 6.

3.1.4. Effect of MS medium supplemented with different concentration of BAP and 0.5 mg/l IAA on shoot induction from nodal explants:

Different concentrations of BAP (5.0 to 10.0 mg/l) and 0.5 mg/l IAA were supplemented with MS media. Results of these observations are presented in **Table-4**. 30 explants were inoculated; shoot initiation started from 16 to 19 days, 19 to 26 explants showed shoot initiation. Mean number of multiple shoot ranges from 2 to 4. Mean length of plantlet ranges from 1 to 2.5 cm. Mean size of leaf is recorded 1.0 cm and 9 to 14 mean numbers of leaves were found. Highest of this combination, 86.67% of explant showed regeneration from MS medium supplemented with 8.0, 9.0, 10.0 mg/l BAP and 0.5 mg/l IAA, and lowest 63.33% of explant showed regeneration from MS medium supplemented with 5.0 mg/l BAP and 0.5 mg/l IAA. Highest mean number of multiple shoot 4, mean length of plantlet 2.5 cm comes from media containing 5.0 and 6.0 mg/l BAP and 0.5 mg/l IAA, lowest mean number of multiple shoot 2, mean length of plantlet 1 cm comes from media containing 10.0 mg/l BAP and 0.5 mg/l IAA. Highest leaves numbers 14 and lowest leaves numbers 9 recorded from media containing 9.0 and 7.0 mg/l BAP with 0.5 mg/l IAA respectively.

3.1.5. Effect of MS medium supplemented with different concentration of BAP and 0.5 mg/l GA_3 on shoot induction from nodal explant:

Different concentrations of BAP (5.0 to 10.0 mg/l) and 0.5 mg/l GA_3 were supplemented with MS media. Results of these observations are presented in **Table-5**. 30 explants were inoculated; shoot initiation started from 12 to 14 days, 22 to 24 explants showed shoot initiation. Mean number of multiple shoot ranges from 2 to 4. Mean length of plantlet 2.5 cm. Mean size of leaf is recorded from 1.0 to 1.5 cm and 12 to 14 mean numbers of leaves were found.

3.1.6. Effect of MS medium supplemented with different concentration of BAP, 0.5 mg/l Kn and 0.5 mg/l NAA on shoot induction from nodal explant:

Different concentrations of BAP (5.0 to 10.0 mg/l), 0.5 mg/l Kn and 0.5 mg/l NAA were supplemented with MS media. Results of these observations are presented in **Table-6.** 30 explants were inoculated; shoot initiation started from 16 to 18 days, 23 to 27 explants showed shoot initiation. Mean number of multiple shoot ranges from 2 to 5. Mean length of plantlet ranges from 2 to 4 cm. Mean size of leaf is recorded from 1.0 to 1.5 cm and 10 to 14 mean number of leaf were found. Highest of this combination, 90% of explant showed regeneration from MS medium supplemented with 8.0, 9.0, 10.0 mg/l BAP, 0.5 mg/l Kn and 0.5 mg/l NAA, and lowest 76.67% of explant showed regeneration from MS medium supplemented with 5.0 mg/l BAP, 0.5 mg/l Kn and 0.5 mg/l NAA. Highest mean number of multiple shoot 5, mean length of plantlet 4 cm, mean size of leaf 1.5 cm and mean numbers of leaves 14, comes from media containing 9.0 mg/l BAP 0.5mg/l Kn and 0.5 mg/l NAA, lowest mean number of multiple shoot 2, mean length of plantlet 2.0 cm mean size of leaf 1.0 cm and mean numbers of leaves 10, comes from media containing 5.0 mg/l BAP 0.5 mg/l Kn and 0.5 mg/l NAA.

3.1.7. Effect of MS medium supplemented with different concentration of BAP, 0.5 mg/l IAA and 0.5 mg/l NAA on shoot induction from nodal explant:

Different concentrations of BAP (5.0 to 10.0 mg/l), 0.5 mg/l IAA and 0.5 mg/l NAA were supplemented with MS media. Results of these observations are presented in **Table-7**. 30 explants were inoculated; shoot initiation started from 12 to 14 days, 20 to 22 explants showed shoot initiation. Mean number of multiple shoot ranges from 2

to 5. Mean length of plantlet ranges from 2.5 to 4 cm. Mean size of leaf is recorded from 1.0 to 2.0 cm and 10 to 14 mean numbers of leaves were found.

3.1.8. Effect of MS medium supplemented with different concentration of BAP, 0.5 mg/l IAA and 0.5 mg/l Kn on shoot induction from nodal explant:

Different concentrations of BAP (5.0 to 10.0 mg/l), 0.5 mg/l IAA and 0.5 mg/l Kn were supplemented with MS media. Results of these observations are presented in **Table-8**. 30 explants were inoculated; shoot initiation started from 12 to 14 days, 20 to 24 explants showed shoot initiation. Mean number of multiple shoot ranges from 3 to 5. Mean length of plantlet ranges from 2 to 3 cm. Mean size of leaf is recorded 1.0 cm and 10 to 12 mean numbers of leaves were found. Best result of this combination was found from MS media supplemented with 5.0 mg/l BAP, 0.5 mg/l IAA and 0.5 mg/l Kn, 24 of responsive explant, 80% explant showed regeneration, 12 days required for initiation of shooting, mean number of multiple shoot 5, mean length of plantlet 3 cm, mean size of leaf was 1.0 cm and mean numbers of leaves were 12. Poor result of this combination was found from MS media supplemented with 10.0 mg/l BAP, 0.5 mg/l IAA and 0.5 mg/l Kn, 20 of responsive explant, 66.67% explant showed regeneration, 14 days required for initiation of shooting, mean number of multiple shoot 3, mean length of plantlet 2.0 cm, mean size of leaf is 1.0 cm and mean numbers of leaves were 10.

3.1.9. Effect of MS medium supplemented with different concentration of BAP, 0.5 mg/l GA_3 and 0.5 mg/l IAA on shoot induction from nodal explant:

Different concentrations of BAP (5.0 to 10.0 mg/l), 0.5 mg/l GA_3 and 0.5 mg/l IAA were supplemented with MS media. Results of these observations are presented in **Table-9**. 30 explants were inoculated; shoot initiation started from 13 to 17 days, 22 to 24 explants showed shoot initiation. Mean number of multiple shoot ranges from 2 to 3. Mean length of plantlet ranges from 2.5 to 3.0 cm. Mean size of leaf is recorded 1.5 cm and 10 to 12 mean numbers of leaves were found.

3.1.10. Effect of MS medium supplemented with different concentration of BAP, 0.5 mg/l GA_3 and 0.5 mg/l Kn on shoot induction from nodal explant:

Different concentrations of BAP (5.0 to 10.0 mg/l), 0.5 mg/l GA_3 and 0.5 mg/l Kn were supplemented with MS media. Results of these observations are presented in

Table-10. 30 explants were inoculated; shoot initiation started from 14 to 17 days, 22 to 23 explants showed shoot initiation. Mean number of multiple shoot was 2. Mean length of plantlet 2.5 cm. Mean size of leaf is recorded 1.0 cm and 10 to 12 mean numbers of leaves were found.

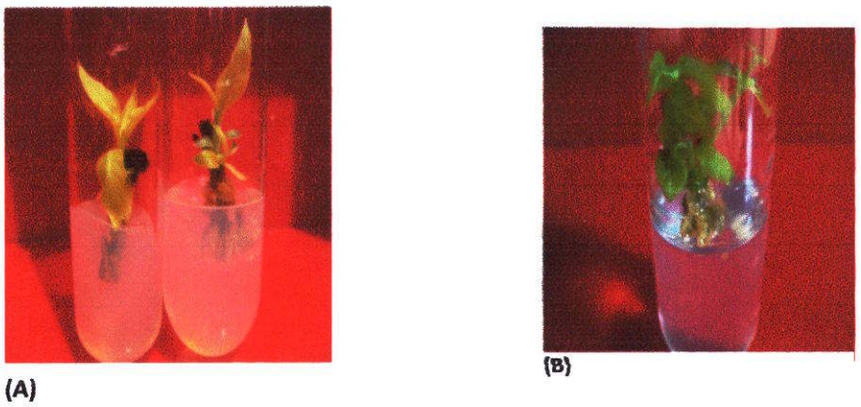


Figure-5: Regeneration from nodal explants of *Adhatoda vasica.*(A) Before first subculture (B) After subculture.

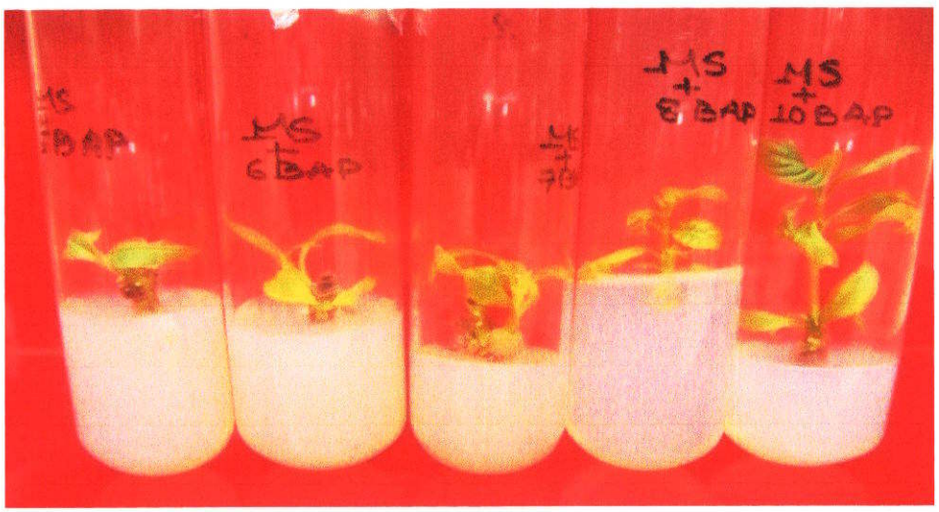
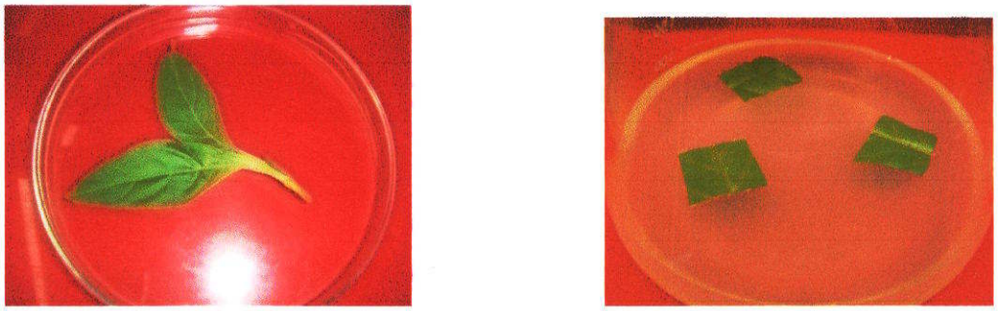


Figure-6: Comparative response of *Adhatoda vasica* on MS medium supplemented with 6.0, 7.0, 8.0, 9.0 and 10.0 mg/l BAP respectively

Figure 7: Regenerated shoot of *Adhatoda vasica*

3.2. Callus Formation from Leaves:

Induction of callus from fresh young leaves and shoot regeneration from the callus were performed. The experiment was carried out to investigate the callus induction ability of the leaves of *Adhatoda vasica*. Leaf explants were cultured on agar solidified MS media supplemented with 2-4-D, IAA, NAA, BAP, IBA, GA_3 and Kn singly or in combinations (Fig.-8). After inoculation leaf explants become enlarged and started to induce callus (Fig.-9A & 9B).



(A) (B)

Figure-8: Explant forn callus induction. (A) Leaf explant (B) excised leaf inoculated on agar solidified MS media supplemented with hormones.

Days required for callus initiation, number of explants showed callus formation, their percentage and the colour of callus were recorded. Results of these observations were recorded after 30 and 60 days from inoculation are presented at next:

3.2.1. Effect of different concentrations of BAP and 2-4-D on callus induction of leaf explants:

Two concentrations of BAP (0.0 and 1.0 mg/l) and different concentrations of 2-4-D (2.0 - 5.0 mg/l) were used with MS media. Results of these observations are presented in **Table-11**. 30 explants were inoculated; callus initiation started from 14 to 24 days,

18 to 26 explants showed callus formation. Best response from this combination was come from media containing 1.0 mg/l BAP and 5.0 mg/l 2-4-D; callus initiation started from 14 days, 26 explants showed callus formation, 86.67% of responsive explants and callus gives light green colour. Poor response from this combination was come from media containing 2.0 mg/l 2-4-D without BAP; callus initiation started from 24 days, 18 explants showed callus formation, 60% of responsive explants and callus gives white colour.

3.2.2. Effect of different concentrations of BAP and 2.0 mg/l 2-4-D on callus induction of leaf explants:

Different concentrations of BAP (3.0 to 7.0 mg/l) and 2.0 mg/l 2-4-D were used with MS media. Results of these observations are presented in **Table-12.** 30 explants were inoculated; callus initiation started from 20 to 23 days, 19 to 28 explants showed callus formation. Well response from this combination was come from media containing 7.0 mg/l BAP and 2.0 mg/l 2-4-D; callus initiation started from 20 days, 28 explants showed callus formation, 93.33% of responsive explants but its callus gave white colour. In media containing 6.0 mg/l BAP and 2.0 mg/l 2-4-D; callus initiation started from 20 days, 23 explants showed callus formation, 76.37% of responsive explants and callus gives light green colour. Poor response from this combination was come from media containing 2.0 mg/l 2-4-D with 3.0 mg/l BAP; callus initiation started from 23 days, 19 explants showed callus formation, 63.33% of responsive explants and callus gives white colour.

3.2.3. Effect of different concentrations of Kn with 3.0 mg/l 2-4-D on callus induction of leaf explants:

Different concentrations of Kn (0.5 to 2.0 mg/l) with 3.0 mg/l 2-4-D were used with MS media. Results of these observations are presented in **Table-13.** 30 explants were inoculated; callus initiation started from 16 to 19 days, 20 to 26 explants showed callus formation. Well response from this combination was come from media containing 2.0 mg/l Kn and 3.0 mg/l 2-4-D; callus initiation started from 16 days, 26 explants showed callus formation, 86.67% of responsive explants and callus gives light green colour. Poor response from this combination was come from media containing 0.5 mg/l Kn with 3.0 mg/l BAP; callus initiation started from 19 days, 20

explants showed callus formation, 66.67% of responsive explants and callus gives light green colour.

3.2.4. Effect of different concentrations of 2-4-D with 1.0 mg/l Kn on callus induction of leaf explants:

Different concentrations of 2-4-D (3.5 to 5.5 mg/l) with 1.0 mg/l Kn were used with MS media. Results of these observations are presented in **Table-14**. 30 explants were inoculated; callus initiation started from 20 to 24 days, 13 to 20 explants showed callus formation. Well response from this combination was come from media containing 5.5 mg/l 2-4-D and 1.0 mg/l Kn; 20 explants showed callus formation, 66.67% of responsive explants and callus gives light green colour but callus initiation started from 24 days. Poor response from this combination was come from media containing 3.5 mg/l 2-4-D and 1.0 mg/l Kn; callus initiation started from 21 days, 13 explants showed callus formation, 43.33% of responsive explants and callus gives light green colour.

3.2.5. Effect of different concentrations of 2-4-D, BAP and Kn on callus induction of leaf explants:

Different concentrations of 2-4-D (2.0 to 5.0 mg/l), BAP (1.0 to 4.0)mg/l and Kn (1.0 to2.0) mg/l were used with MS media. Results of these observations are presented in **Table-15**. 30 explants were inoculated; callus initiation started from 11 to 14 days, 10 to 18 explants showed callus formation. Well response from this combination was come from media containing 2.0 mg/l 2-4-D, 2.0 mg/l BAP and 2.0 mg/l Kn; callus initiation started from 12 days, 18 explants showed callus formation, 60% of responsive explants and callus gives light green colour. Poor response from this combination was come from media containing 2.5, 3.5, 4.0 mg/l 2-4-D, 2.0, 2.0, 4.0 mg/l BAP with 1.0 mg/l Kn; callus initiation started from 11 days, 10 explants showed callus formation, 33.33% of responsive explants and callus gives light green colour.

3.2.6. Effect of different concentrations of GA_3 with 0.05 mg/l IAA on callus induction of leaf explants:

Different concentrations of GA_3 (0.5 to 2.5 mg/l) with 0.05 mg/l IAA were used with MS media. Results of these observations are presented in **Table-16**. 30 explants were

inoculated; callus initiation started from 23 to 26 days, 20 to 25 explants showed callus formation. Well response from this combination was come from media containing 2.5 mg/l GA_3 and 0.05 mg/l IAA; callus initiation started from 23 days, 25 explants showed callus formation, 83.33% of responsive explants and callus gives light green colour. Poor response from this combination was come from media containing 0.5 and 1.0 mg/l GA_3 with 0.05 mg/l IAA; callus initiation started from 26 days, 20 explants showed callus formation, 66.67% of responsive explants and callus gives light green colour.

3.2.7. Effect of different concentrations of BAP with 1.0 mg/l GA_3 on callus induction of leaf explants:

Different concentrations of BAP (0.05 to 0.09 mg/l) with 1.0 mg/l GA_3 were used with MS media. Results of these observations are presented in **Table-17**. 30 explants were inoculated; callus initiation started from 29 to 30 days, 12 to 17 explants showed callus formation. High response from this combination was come from media containing 0.09 mg/l BAP and 1.0 mg/l GA_3; callus initiation started from 29 days, 17 explants showed callus formation, 56.67% of responsive explants and callus gives white colour. Low response from this combination was come from media containing 0.05 and 0.06 mg/l BAP with 1.0 mg/l GA_3; callus initiation started from 30 days, 12 explants showed callus formation, 40% of responsive explants and callus gives yellowish white colour.

3.2.8. Effect of different concentrations of GA_3 with 1.0 mg/l BAP on callus induction of leaf explants:

Different concentrations of GA_3 (0.05 to 0.09 mg/l) with 1.0 mg/l BAP were used with MS media. Results of these observations are presented in **Table-18**. 30 explants were inoculated; callus initiation started from 19 to 23 days, 24 to 26 explants showed callus formation. High response from this combination was come from media containing 0.09 mg/l GA_3 and 1.0 mg/l BAP; callus initiation started from 19 days, 26 explants showed callus formation, 86.67% of responsive explants and callus gives light green colour. Low response from this combination was come from media containing 0.05 and 0.06 mg/l GA_3 and 1.0 mg/l BAP; callus initiation started from 23 days, 24 explants showed callus formation, 80% of responsive explants and callus gives light green colour.

3.2.9. Effect of different concentrations of NAA with 1.0 mg/l BAP on callus induction of leaf explants:

Different concentrations of NAA (0.05 to 0.09 mg/l) with 1.0 mg/l BAP were used with MS media. Results of these observations are presented in **Table-19**. 30 explants were inoculated; callus initiation started from 25 to 27 days, 13 to 20 explants showed callus formation. High response from this combination was come from media containing 0.09 mg/l NAA and 1.0 mg/l BAP; callus initiation started from 25 days, 20 explants showed callus formation, 66.67% of responsive explants and callus gives light green colour. Low response from this combination was come from media containing 0.07 mg/l NAA with 1.0 mg/l BAP; callus initiation started from 27 days, 13 explants showed callus formation, 43.33% of responsive explants and callus gives light green colour.

3.2.10. Effect of different concentrations of BAP with 1.0 mg/l NAA on callus induction of leaf explants:

Different concentrations of BAP (0.5 to 2.5 mg/l) with 1.0 mg/l NAA were used with MS media. Results of these observations are presented in **Table-20**. 30 explants were inoculated; callus initiation started from 22 to 25 days, 19 to 20 explants showed callus formation. . Response from this combination was medium, media containing 0.5, 1.0 and 1.5 mg/l BAP with 1.0 mg/l NAA showed starting of callus initiation from 21, 22, 24 days respectively, 20 explants showed callus formation, 66.67% of responsive explants and callus gives white colour. Media containing 2.0 and 2.5 mg/l BAP with 1.0 mg/l NAA showed starting of callus initiation from 25 days, 19 explants showed callus formation, 63.33% of responsive explants and callus gives white colour.

3.2.11. Effect of different concentrations of NAA with 1.0 mg/l IBA on callus induction of leaf explants:

Different concentrations of NAA (0.5 to 2.5 mg/l) with 1.0 mg/l IBA were used with MS media. Results of these observations are presented in **Table-21**. 30 explants were inoculated; callus initiation started from 27 to 29 days, 16 to 17 explants showed callus formation. Response from this combination was medium, media containing 0.5 and 1.0 mg/l NAA with 1.0 mg/l IBA showed starting of callus initiation from 29 days, 17 explants showed callus formation, 56.67% of responsive explants and callus

gives light green colour. Media containing 1.5, 2.0 and 2.5 mg/l NAA with 1.0 mg/l IBA showed starting of callus initiation from 29, 28, 27 days respectively, 16 explants showed callus formation, 53.33% of responsive explants and callus gives light green colour.

3.2.12. Effect of different concentrations of IAA alone and in combination with NAA or BAP or GA_3 on callus induction of leaf explants:

Two concentration of IAA (0.05 and 1.0 mg/l) alone and in combination with NAA or BAP or GA_3 were used with MS media. Results of these observations are presented in **Table-22**. Highest result of all combinations comes from media containing 0.05 mg/l IAA, 0.05 mg/l NAA and 1.0 mg/l BAP. 30 explants were inoculated; callus initiation started from 14 days, 30 explants showed callus formation, 100% of responsive explants and callus gives green colour. Lowest result of IAA combination obtained when 0.05 mg/l IAA alone was used, callus initiation started from 26 days, 7 explants showed callus formation, 23.33% of responsive explants and callus gives white colour.

3.2.13. Effect of different concentrations of IAA alone and in combination with NAA or BAP or GA_3 on callus induction of leaf explants:

Two concentration of IBA (0.05 and 1.0 mg/l) alone and in combination with NAA or BAP or GA_3 were used with MS media. Results of these observations are presented in **Table-23**. Highest result of IBA combination comes from media containing 1.0 mg/l IBA with 1.0 mg/l GA_3. 30 explants were inoculated; callus initiation started from 14 days, 28 explants showed callus formation, 93.33% of responsive explants and callus gives light green colour. Lowest result obtained when 0.05 mg/l IBA alone was used, callus initiation started from 28 days, 10 explants showed callus formation, 33.33% of responsive explants and callus gives white colour.

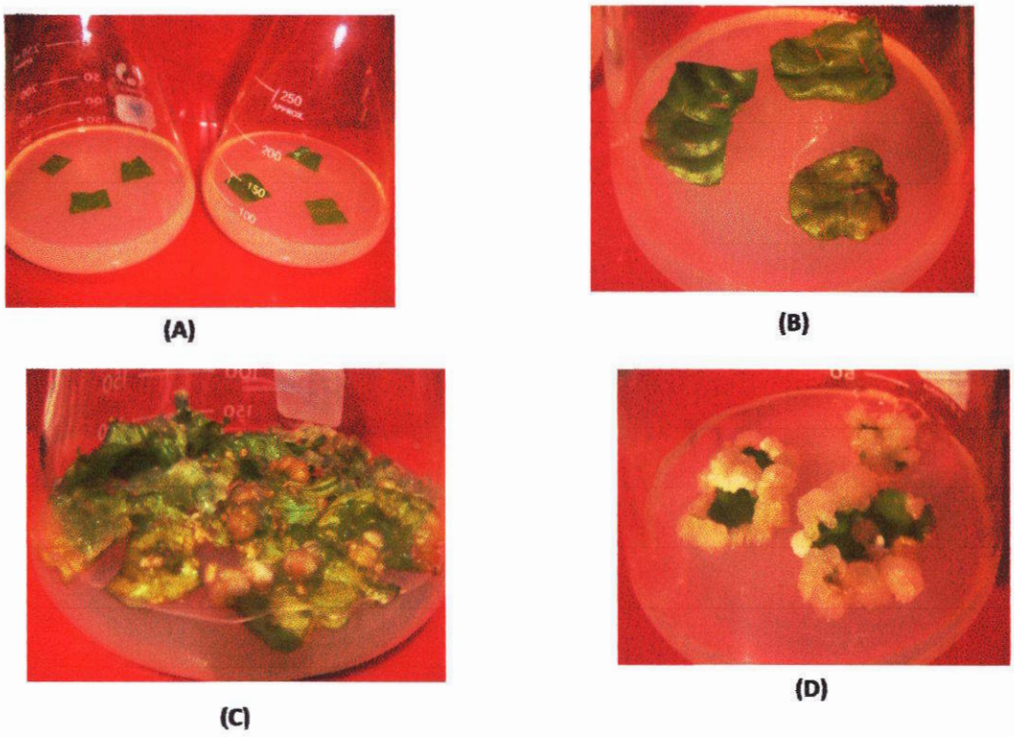


Figure-9: Callus induction from *Adhatoda vasica* leaves. (A) Small excised leaf explant at the day of inoculation, (B) leaves become enlarged and initiation of callus induction. (C) Green callus on IAA (0.05mg/l) + BAP (1.0 mg/l) medium. (D) Light green callus on IBA (1.0 mg/l) + GA_3 (1.0 mg/l) medium.

3.3. Callus-mediated shoot regeneration of *Adhatoda vasica*:

Light green to green callus are well proliferative and good for regeneration than white callus. These callus were sub-cultured on the same media after 7 days. At least after three subcultures, vigorous callus were transferred on the shooting medium. MS media supplemented with Different concentration of BAP were used for shooting from callus.

3.3.1. Effect of MS medium supplemented with different concentration of BAP on shoot induction from callus:

Different concentrations of BAP (0.5 to 10.0 mg/l) were supplemented with MS media. Results of this observation are presented in **Table-24**. 10 callus were inoculated; shoot initiation started from 10 to21 days, 4 to 8 callus showed shoot initiation, mean number of multiple shoot ranges from 2 to 4, Mean length of plantlet ranges from 1.5 to 3.0 cm. Mean size of leaf is recorded from 0.6 to 1.0 cm and 5 to 11 mean number of leaf were found. Best result was found from MS+10.0 mg/l BAP

medium, 8 of responsive explant, 80% explant showed regeneration, 10 days required for initiation of shooting, mean number of multiple shoot 4, mean length of plantlet 3.0 cm, mean size of leaf is 1.0 cm and mean number of leaf were 11. Poor result was found from MS+0.5 mg/l BAP medium, 4 of responsive explant, 40% explant showed regeneration, 21 days required for initiation of shooting, mean number of multiple shoot 2, mean length of plantlet 1.5 cm, mean size of leaf is 0.6 cm and mean number of leaf were 5.

Figure-10: Multiple Shoot regeneration from callus cells of *Adhatoda vasica.*

3.4. *In vitro* Initiation of rooting of *Adhatoda vasica*:

Shoot induced from both callus and regeneration from nodal explants were subcultured for 3 (three) times in same shooting medium each after 2 (two) weeks then transferred into rooting medium. MS, ½ MS, MS + 1.0 mg/l IBA, ½ MS, MS + 1.0 mg/l IBA & MS + 2.5 mg/l IBA + 0.5 mg/l IAA+0.5mg/l NAA were prepared as rooting medium. after 30 and 60 observation result was taken.

3.4.1. Effect of different Media Concentrations and supplements on Root initiation of Shoot Induced from Nodal Explants:

Different media concentrations (MS + ½ MS) alone and in combination with supplements (IBA, NAA & IAA) in different concentrations were applied for in vitro initiation of rooting of Adhatoda vasica shoot induced from nodal explant. Results of this observations are presented in **Table-25**. 10 explants were inoculated; 3 to 7 explants showed initiation of rooting that is, 30-80% of responsive explants, root initiation started from 12 to 21 days. best result was obtained from MS medium containing 2.5mg/l IBA,0.5 m/l NAA **(Fig.-11)**. 8 explants showed initiation of rooting. That is 80% of responsive explants, root initiation started from 12 days. MS, ½ MS, & MS, ½ MS with 1.0 mg/l IBA showed poor and moderate result respectively.

Figure- 11: Rooting of shoot regenerated from nodal explants of *Adhatoda vasica*

3.4.2. Effect of different Media Concentrations and supplements on Root initiation of Shoot Induced from Callus:

Different media concentrations (MS + ½ MS) alone and in combination with supplements (IBA, NAA & IAA) in different concentrations were applied for *in vitro* initiation of rooting of Adhatoda vasica shoot induced from callus. Results of this observations are presented in **Table-26**. 5 explants were inoculated; 1 to 3 explants showed initiation of rooting that is, 20-60% of responsive explants, root initiation started from 12 to 22 days. Best result was obtained from MS medium containing 2.5 mg/l IBA,0.5 mg/l IAA & 0.5 mg/l NAA **(Fig.-12)**. 3 explants showed initiation of rooting. That is 60% of responsive explants, root initiation started from 13 days. MS, ½ MS, & MS, ½ MS with 1.0 mg/l IBA showed poor and moderate result respectively.

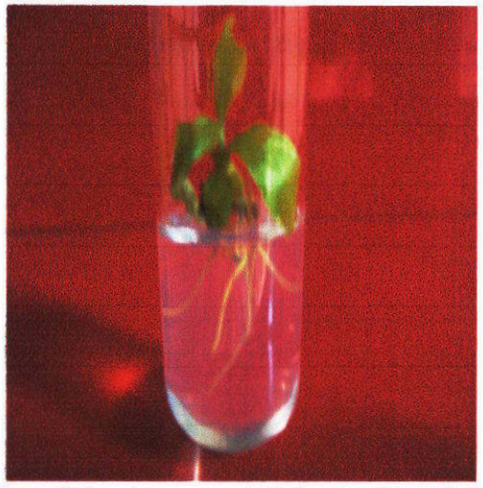


Figure-12: Rooting of shoot regenerated from callus of *Adhatoda vasica*

3.5. Transplantation of *in vitro* Grown *Adhatoda vasica* Plantlets on soil:

After initiation of roots these plantlets were taken out from the growth room, at least 25-30 days old rooted plantlets were chosen for this purpose and were kept under normal temperature for about two days in contact with normal atmospheric temperature and light. After 2 days the plantlets were taken out from culture vessels and washed with running tap water and afterwards transferred to plastic pots containing soil and then covered with polythene bags to prevent desiccation. Plantlets on plastic pots were taken out from indoor and kept under normal condition. After three or four days polythene bags were removed **(Fig.-13)**. Well survived plants were able to transfer after 14 days on garden soil. the ultimate survival rate of the transferred plantlets to soil was about 80% and their growth in such condition was satisfactory. Mortality of about 20% occurred during transplantation of plantlets due to shifting between containers, injuries to the root system and excessive evaporation.

Figure- 13: Hardening of *Adhatoda vasica* plantlets on plastic pot.

Table-4. Effect of MS medium supplemented with different concentration of BAP and 0.5 mg/l IAA on shoot induction from nodal explant of *Adhatoda vasica* (Basok) in 30 days.

Supplements (mg/l)		No. of responsive explant	% of explant showed regeneration	Days required for initiation	Multiple shoot (mean)	Length of plantlet (cm)	Size of leaf (cm)	No. of leaf
IAA	BAP							
0.5	5.0	19	63.33	16	4	2.5	1	10
0.5	6.0	21	70	17	4	2.5	1	10
0.5	7.0	23	76.67	19	2	2	1	9
0.5	8.0	26	86.67	19	2	2	1	12
0.5	9.0	26	86.67	19	3	1	1	14
0.5	10.0	26	86.67	19	2	1	1	12

Table-5. Effect of MS medium supplemented with different concentration of BAP and 0.5 mg/l GA_3 on shoot induction from nodal explant of *Adhatoda vasica* (Basok) in 30 days.

Supplements (mg/l)		No. of responsive explant	% of explant showed regeneration	Days required for initiation	Multiple shoot (mean)	Length of plantlet (cm)	Size of leaf (cm)	No. of leaf
GA_3	BAP							
0.5	5.0	23	76.67	12	4	2.5	1	12
0.5	6.0	23	76.67	14	4	2.5	1.5	12
0.5	7.0	24	80	14	3	2.5	1.5	12
0.5	8.0	23	76.67	14	3	2.5	1.5	14
0.5	9.0	22	73.33	13	2	2.5	1.5	14
0.5	10.0	22	73.33	13	2	2.5	1.5	14

Table-6. Effect of MS medium supplemented with different concentration of BAP, 0.5 mg/l Kn and 0.5 mg/l NAA on shoot induction from nodal explant of *Adhatoda vasica* (Basok) in 30 days.

Supplements (mg/l)			No. of responsive explant	% of explant showed regeneration	Days required for initiation	Multiple shoot (mean)	Length of plantlet (cm)	Size of leaf (cm)	No. of leaf
Kn	BAP	NAA							
0.5	5.0	0.5	23	76.67	16	2	2	1	10
0.5	6.0	0.5	24	80	17	2	3	1	12
0.5	7.0	0.5	26	86.67	18	3	3	1	12
0.5	8.0	0.5	27	90	16	4	2	1.5	13
0.5	9.0	0.5	27	90	16	5	4	1.5	14
0.5	10.0	0.5	27	90	16	5	4	1.5	11

Table-7. Effect of MS medium supplemented with different concentration of BAP, 0.5 mg/l IAA and 0.5 mg/l NAA on shoot induction from nodal explant of *Adhatoda vasica* (Basok) in 30 days.

Supplements (mg/l)			No. of responsive explant	% of explant showed regeneration	Days required for initiation	Multiple shoot (mean)	Length of plantlet (cm)	Size of leaf (cm)	No. of leaf
IAA	BAP	NAA							
0.5	5.0	0.5	22	73.33	13	2	2.5	1.5	10
0.5	6.0	0.5	22	73.33	14	3	2.8	1.5	10
0.5	7.0	0.5	21	70	13	3	3.0	2	11
0.5	8.0	0.5	20	66.67	13	4	3.5	1.5	13
0.5	9.0	0.5	20	66.67	13	5	4.0	1	14
0.5	10.0	0.5	21	70	12	4	3.6	1	14

Table-8. Effect of MS medium supplemented with different concentration of BAP, 0.5 mg/l IAA and 0.5 mg/l Kn on shoot induction from nodal explant of *Adhatoda vasica* (Basok) in 30 days.

Supplements (mg/l)			No. of responsive explant	% of explant showed regeneration	Days required for initiation	Multiple shoot (mean)	Length of plantlet (cm)	Size of leaf (cm)	No. of leaf
IAA	BAP	Kn							
0.5	5.0	0.5	24	80	12	5	3	1	12
0.5	6.0	0.5	24	80	13	4	3	1	10
0.5	7.0	0.5	24	80	14	4	2.5	1	10
0.5	8.0	0.5	21	70	14	4	2.5	1	11
0.5	9.0	0.5	20	66.67	14	4	2.5	1	12
0.5	10.0	0.5	20	66.67	14	3	2	1	10

Table-9. Effect of MS medium supplemented with different concentration of BAP, 0.5 mg/l GA_3 and 0.5 mg/l IAA on shoot induction from nodal explant of *Adhatoda vasica* (Basok) in 30 days.

Supplements (mg/l)			No. of responsive explant	% of explant showed regeneration	Days required for initiation	Multiple shoot (mean)	Length of plantlet (cm)	Size of leaf (cm)	No. of leaf
IAA	BAP	GA_3							
0.5	5.0	0.5	24	80	16	3	2.5	1.5	10
0.5	6.0	0.5	24	80	17	3	3	1.5	12
0.5	7.0	0.5	23	76.67	14	3	3	1.5	12
0.5	8.0	0.5	22	73.33	13	3	3	1.5	11
0.5	9.0	0.5	22	73.33	13	2	3	1.5	11
0.5	10.0	0.5	22	73.33	13	2	3	1.5	11

Table-10. Effect of MS medium supplemented with different concentration of BAP, 0.5 mg/l GA_3 and 0.5 mg/l Kn on shoot induction from nodal explant of *Adhatoda vasica* (Basok) in 30 days.

Supplements (mg/l)			No. of responsive explant	% of explant showed regeneration	Days required for initiation	Multiple shoot (mean)	Length of plantlet (cm)	Size of leaf (cm)	No. of leaf
GA_3	BAP	Kn							
0.5	5.0	0.5	23	76.67	16	2	2.5	1	10
0.5	6.0	0.5	23	76.67	17	2	2.5	1	10
0.5	7.0	0.5	23	76.67	14	2	2.5	1	11
0.5	8.0	0.5	22	73.33	14	2	2.5	1	11
0.5	9.0	0.5	22	73.33	15	2	2.5	1	11
0.5	10.0	0.5	22	73.33	16	2	2.5	1	12

Table-11. Effect of different concentrations of BAP and 2-4-D on callus induction of leaf explants of *Adhatoda vasica* (Basok) in 30 days.

Supplements (mg/l)		No. of explants inoculated	Days required for initiation of callus	No. of explants showed callus formation	% of responsive explants	Colour
BAP	2-4-D					
-	2.0	30	24	18	60	White
1.0	2.0	30	20	21	70	White
1.0	3.0	30	18	23	76.67	White
1.0	4.0	30	18	23	76.67	Light green
1.0	5.0	30	14	26	86.67	Light green

Table-12. Effect of different concentrations of BAP and 2.0 mg/l 2-4-D on callus induction of leaf explants of *Adhatoda vasica* (Basok) in 30 days.

Supplements (mg/l)		No. of explants inoculated	Days required for initiation of callus	No. of explants showed callus formation	% of responsive explants	Colour
2-4-D	BAP					
2.0	3.0	30	23	19	63.33	White
2.0	4.0	30	23	21	70	White
2.0	5.0	30	20	21	70	White
2.0	6.0	30	20	23	76.67	Light green
2.0	7.0	30	20	28	93.33	White

Table-13. Effect of different concentrations of Kn with 3.0 mg/l 2-4-D on callus induction of leaf explants of *Adhatoda vasica* (Basok) in 30 days.

Supplements (mg/l)		No. of explants inoculated	Days required for initiation of callus	No. of explants showed callus formation	% of responsive explants	Colour
2-4-D	Kn					
3.0	0.5	30	19	20	66.67	Light green
3.0	0.5	30	17	20	66.67	Light green
3.0	1.0	30	17	25	83.33	Light green
3.0	1.0	30	17	26	86.67	Light green
3.0	2.0	30	16	26	86.67	Light green

Table-14. Effect of different concentrations of 2-4-D with 1.0 mg/l Kn on callus induction of leaf explants of *Adhatoda vasica* (Basok) in 30 days.

Supplements (mg/l)		No. of explants inoculated	Days required for initiation of callus	No. of explants showed callus formation	% of responsive explants	Colour
Kn	2-4-D					
1.0	3.5	30	21	13	43.33	Light green
1.0	4.0	30	23	15	50	Light green
1.0	4.5	30	22	15	50	Light green
1.0	5.0	30	20	18	60	Light green
1.0	5.5	30	24	20	66.67	Light green

Table-15. Effect of different concentrations of 2-4-D, BAP and Kn on callus induction of leaf explants of *Adhatoda vasica* (Basok) in 30 days.

Supplements (mg/l)			No. of explants inoculated	Days required for initiation of callus	No. of explants showed callus formation	% of responsive explants	Colour
2-4-D	BAP	Kn					
2.0	1.0	1.0	30	14	13	43.33	Light green
3.0	1.0	1.0	30	14	13	43.33	Light green
4.0	1.0	1.0	30	14	14	46.67	Light green
5.0	1.0	1.0	30	14	14	46.67	Light green
2.0	2.0	2.0	30	12	18	60	Light green
2.5	2.0	1.0	30	11	10	33.33	Light green
3.5	2.0	1.0	30	11	10	33.33	Light green
4.0	4.0	1.0	30	11	10	33.33	Light green

Table-20. Effect of different concentrations of BAP with 1.0 mg/l NAA on callus induction of leaf explants of *Adhatoda vasica* (Basok) in 30 days.

Supplements (mg/l)		No. of explants inoculated	Days required for initiation of callus	No. of explants showed callus formation	% of responsive explants	Colour
NAA	BAP					
1.0	0.5	30	21	20	66.67	White
1.0	1.0	30	22	20	66.67	White
1.0	1.5	30	24	20	66.67	White
1.0	2.0	30	25	19	63.33	White
1.0	2.5	30	25	19	63.33	White

Table-21. Effect of different concentrations of NAA with 1.0 mg/l IBA on callus induction of leaf explants of *Adhatoda vasica* (Basok) in 30 days.

Supplements (mg/l)		No. of explants inoculated	Days required for initiation of callus	No. of explants showed callus formation	% of responsive explants	Colour
IBA	NAA					
1.0	0.5	30	29	17	56.67	Light green
1.0	1.0	30	29	17	56.67	Light green
1.0	1.5	30	29	16	53.33	Light green
1.0	2.0	30	28	16	53.33	Light green
1.0	2.5	30	27	16	53.33	Light green

Table-22. Effect of different concentrations of IAA alone and in combination with NAA or BAP or GA_3 on callus induction of leaf explants of *Adhatoda vasica* (Basok) in 30 days.

Supplements (mg/l)				No. of explants inoculated	Days required for initiation of callus	No. of explants showed callus formation	% of responsive explants	Colour
IAA	NAA	BAP	GA_3					
0.05	-	-	-	30	26	7	23.33	White
0.05	-	0.05	-	30	16	10	33.33	White
0.05	0.05	1.0	-	30	14	30	100	Green
0.05		-	1.0	30	17	12	40	Light green
1.0		-	1.0	30	17	10	33.33	Light green
1.0	-	-	-	30	20	14	46.67	White

Table-23. Effect of different concentrations of IAA alone and in combination with NAA or BAP or GA_3 on callus induction of leaf explants of *Adhatoda vasica* (Basok) in 30 days.

Supplements (mg/l)				No. of explants inoculated	Days required for initiation of callus	No. of explants showed callus formation	% of responsive explants	Colour
IBA	NAA	BAP	GA_3					
0.05	-	-	-	30	28	10	33.33	White
1.0	-	-	-	30	27	10	33.33	White
1.0	0.05	-	-	30	21	20	66.67	Light green
1.0	1.0	-	-	30	14	20	66.67	Light green
1.0		-	1.0	30	14	28	93.33	Light green

Table-24. Effect of MS medium supplemented with different concentration of BAP on shoot induction from callus of *Adhatoda vasica* (Basok) in 30 days.

Supplements (mg/l)	No. of responsive explant	% of explant showed re-generation	Days required for initiation	Multiple shoot (mean)	Length of plantlet (cm)	Size of leaf (cm)	No. of leaf (mean)
5.0 BAP	4	40	21	2	1.5	0.6	5
6.0 BAP	5	50	17	2	1.7	0.9	7
7.0 BAP	4	40	19	2	2.0	1	6
8.0 BAP	6	60	16	3	2.5	1	7
9.0 BAP	6	60	12	4	2.7	1	8
10.0 BAP	8	80	10	4	3.0	1	11

Table-25. Effect of different media concentrations and supplements on root initiation of *Adhatoda vasica* shoot induced from nodal explants.

Media/Supplements (mg/l)	No. of explants inoculated	No. of responsive explants	% of explants showed root formation	Days required for initiation of rooting
½MS	10	3	30%	18
MS	10	3	30%	15
½MS+1.0 IBA	10	4	40%	21
MS+1.0 IBA	10	6	60%	14
MS+2.5 IBA+0.5 IAA+0.5 NAA	10	8	80%	12

Table-26. Effect of different media concentrations and supplements on root initiation of *Adhatoda vasica* shoot induced from callus.

Media/Supplements (mg/l)	No. of explants inoculated	No. of responsive explants	% of explants showed root formation	Days required for initiation of rooting
½MS	5	1	20%	22
MS	5	1	20%	17
½MS+1.0 IBA	5	1	20%	16
MS+1.0 IBA	5	2	40%	12
MS+2.5 IBA+0.5 IAA+0.5 NAA	5	3	60%	13

Chapter four

Discussion

4. DISCUSSION

Tissue culture technique involves the establishment of differentiated cell or tissue under a suitable culture conditions, *in vitro* cell proliferation and subsequent regeneration of plants (Scowcroft and Larkin, 1982). In this process firstly the induction of multiple shoot through direct regeneration without an intervening callus phase and secondly the induction of callus from different explants for indirect regeneration through callus is usually done.

In tissue culture techniques, cell totipotency is an important factor. Failure in most plant cell or tissues to get whole plants *in vitro* is due of lack of proper techniques and insufficient knowledge about nutrient media and other physical and chemical conditions, which are essential for proper growth of cells, tissues and organs (Jhori, 1982). Mature tissues are generally less responsive in compared with juvenile tissues (Sommer and Caldas, 1980). The present study is oriented with the use of juvenile tissues like leaf and nodal segment of *Adhatoda vasica* as explant.

Micropropagation has many advantages over conventional methods of vegetative propagation, which suffer from several limitations (Nehra and Kartha, 1994). With micropropagation, the multiplication rate is greatly increased. It also permits the production of contamination free material. Micropropagation of various plant species, including economical and medicinal plants, has been reported (Skirvin, *et al.*, 1990; Withers, 1989; Murashige, 1978). *Adhatoda vasica* has been micropropagated for rapid multiplication using leaf and nodal segments.

It is the backdrop that motivated to undertake the present investigation with a view to develop an efficient protocol for *in vitro* regeneration of plantlets from nodal explants, induction of callus from leaf explants, callus mediated shoot regeneration and root initiation of *Adhatoda vasica*.

4.1. *In vitro* Regeneration of *Adhatoda vasica* from Nodal Explant:

In vitro propagation of plants holds tremendous potential for the production of high quality plants (Murch, *et al.*, 2000). This can be achieved through different methods including micropropagation. Tissue culture technique can be applied, not only to increase propagation rates but also to modify the germplasm itself.

For shoot proliferation, growth regulators especially cytokinins are one of the most important factors that affecting the response of explants (Lane, 1979, Stoltz, 1979, Bhojwani, 1980, Garland and Stolz, 1981). In the present investigation, MS media was used to optimize the shoot proliferation and shoot multiplication of *Adhatoda vasica.* Various hormonal supplements namely, BAP, IAA, NAA, GA_3 and Kn were used singly or in combinations for *in vitro* regeneration of *Adhatoda vasica* from nodal explants.

Among the media components used in this study MS medium supplemented with BAP (10.0 mg/l) was found to be the best for multiple shoot formation, in which 93.33% explants produced multiple shoots, 7 days required for initiation of shooting, The nodal explants remained green and viable for 15 to 20 days, after that the explants were subcultured on the same medium. After 1 month, mean number of multiple shoot were 10.6, mean length of plantlet 5.2 cm, mean size of leaf was 1.8 cm and mean number of leaves were 15.6.whilst the maximum number of shoots of *Adhatoda vasica* 7.75 ± 0.392 differentiated from split nodal halves MS medium supplemented with BAP (10mg/l) during 4 weeks of culture, but these shoots failed to grow upon subculture in the same medium was reported (Gauri A. & V.D. Reddy, 2007). MS medium supplemented with coconut water 15% (v/v) + BAP (5.0 mg/l) started proliferated multiple shoots of *Adhatoda vasica* in 3 to 4 weeks and shoots became 3 cm long in 6 to 8 weeks, was reported best (Raageeva bimal & Shahnawaz, 2012).

MS medium supplemented with (BAP 0.5 to 4.0 mg/l) showed very poor result, succulent shoot and callusing tendency at the base of explants were observed and some cultured explants were turned black, so that next studies were performed with BAP (5.0 to10.0 mg/l) in combination of other hormones. Shoot formation occurred in the MS media supplemented with BAP (2.0 mg/l), 96.67% of shoot developed callus at the cut basal end and the explants turned brown and necrotic due to phenolic exudates released into the medium (Sangeeta N. and Alak K.B., 2005). But the MS medium supplemented with BAP (1.0 mg/l) was reported most suitable medium for *in vitro* establishment of nodal explants. In this medium 2.5±0.2 shoots per explants, 6.2±0.3cm average shoot length and 6.0±0.2 nodes per shoot after 30 days (Prerna Soni, *et al.* 2012).

The nodal explants cultured on MS medium supplemented with Kn, NAA, IAA, GA_3 (0.5 mg/l) each with BAP (5to10 mg/l) showed moderate response (Sangeeta N. and Alak K.B., 2005). Among these Kn (0.5 mg/l) + GA_3 (0.5 mg/l) + BAP (5.0 to10.0 mg/l) & IAA (0.5 mg/l) + NAA (0.5 mg/l) + BAP (5.0 to10.0 mg/l) showed comparatively poor response in explant regeneration and some of these explants were no longer able to establish regeneration after subculture. MS + NAA (0.1 to 1.0 mg/l) + BAP (1.0 to 2.5 mg/l) did not show any morphogenetic response and turned dark in 12 to 15 days (Raageeva bimal & M.d. Shahnawaz, 2012). Kn and BAP promoted shoot proliferation but BAP proved better (Aggarwal and Barna, 2004).

In this experiment, Kn (0.5 mg/l) + NAA (0.5 mg/l), IAA (0.5 mg/l) + NAA (0.5 mg/l), IAA (0.5 mg/l) + Kn (0.5 mg/l), IAA (0.5 mg/l) + GA_3 (0.5 mg/l) + GA_3 (0.5 mg/l) + Kn (0.5 mg/l) each combination with BAP (5.0 to10.0 mg/l) were used which showed moderate result also (Sangeeta N. and Alak K.B., 2005). IAA (0.5 mg/l) + Kn (0.5 mg/l) + BAP (5.0 to10.0 mg/l) showed relatively poor response in regeneration. Some of these explants that do not show regeneration on different media were affected by fungal or bacterial contamination; some were turned black or dried out (Sangeeta N. and Alak K.B., 2005).

Well regenerated shoots were separated and each was subcultured on the same medium and observed that after 3rd subculture the shoot multiplication and other criteria were remained constant (Sangeeta N. and Alak K.B., 2005).

4.2. Formation of Callus from Leaves of *Adhatoda vasica*:

Callus is actively dividing non-organized tissue of undifferentiated and differentiated cells often developing from injury (wounding) or in tissue culture (Pieric, 1987). Callus formed during *in vitro* culture has some similarity to tissue arising *in vivo* injury plants (so called wound callus). However, there is difference in morphology, cellular structure, growth and metabolism between callus derived through tissue culture and natural wound cells. Now it has been established that any tissue can be changed into callus, if cultured on a suitably defined medium under controlled conditions. Callus cells are those cells that cover a plant wound (Takeda, et al., 1981). Callus formation is induced from plant tissues after surface sterilization and plating onto *in vitro* tissue culture medium. Exogenous supply of auxin and often in combination with cytokinin to medium

is essential for callus induction (Min *et al.*, 1991). Interaction of auxins and cytokinins plays vital role in cell division, growth, development, differentiation and the formation of plant organs (Shrivastava and Banerjee, 2008; Purkayastha *et al.*, 2010; Jha *et al.*, 2007). Rao and Lee, (1986) reported that intermediate levels of auxin and cytokinin usually promote callusing. But many other factors such as light, temperature, humidity, etc. are also important for callus production (Pieric, 1987).

In the present investigation, best result among all combinations comes from media containing IAA (0.05 mg/l) + NAA (0.05 mg/l) + BAP (1.0 mg/l). 30 explants were inoculated; callus initiation started from 14 days, 30 explants showed callus formation, 100% of responsive explants and give green colour, soft, fast growing callus. NAA (2.5 mg/l) in combination with BAP (0.5 mg/l) was reported best for callus induction and establishment from nodal segments of *Adhatoda vasica*, (Renu S. and Nidhi B., 2011). Callus induction on BAP and NAA were also observed in *Rauwolfia serpentina* (Tomar and Tiwari, 2006) MS media supplemented with various combinations of auxin and cytokinin, callus formation was observed in 15 and 21 days, respectively (Sunita M. and Dhananjay S., 2010).

Media containing IBA (1.0 mg/l) with GA_3 (1.0 mg/l) also showed better reponse, 30 explants were inoculated; callus initiation started from 14 days, 28 explants showed callus formation, 93.33% of responsive explants and callus gives light green colour. The largest growth of callus occurred on the medium containing IAA (1.5 mg/l)+ GA_3(2.5 mg/l) was reported on spinach (*Spinacia oleracea* L.) (Taha, R.S. *et al,*2010).

2-4-D was used alone or in combination with BAP or Kn or both, in different concentrations to determine callus induction (Sunita M. and Dhananjay S., 2010).Different concentrations of GA_3, IAA, BAP and NAA were used in various combinations. GA_3 (1.0 mg/l) with BAP (0.05-0.09 mg/l) combinations showed very poor response in callus induction, calluses were yellowish white to white, after few days these were turned brown in colour & are non viable (Sunita M. and Dhananjay S., 2010).

Other combinations showed moderate response & formation of viable callus. Callas induction was started but the amount of callus produced was not satisfactory and turned to die after

subsequent subculture (Sunita M. and Dhananjay S., 2010).Light green calluses were found in most of the medium but not all of these remain constant after subculture also, some were dried out, turned brown or black and necrotic due to phenolic exudates released.

Callus induced from the two media IAA (0.05 mg/l) + NAA (0.05 mg/l) + BAP (1.0 mg/l) and IBA (1.0 mg/l) with GA_3 (1.0 mg/l) were showed constant result after 2nd and 3rd subculture, remain viable green or light green calluses and no phenolic exudates released. These calluses were transferred to the shooting medium for regeneration of shoot (Sunita M. and Dhananjay S., 2010).

4.3. Callus-mediated shoot regeneration of *Adhatoda vasica*:

Callus could be excellently induced on appropriate medium. The adventitious shoots were efficiently generated from induced callus. Green and light green calluses were transferred on the medium BAP (0.5-10.0 mg/l) that showed well response in shoot regeneration from nodal explants. Best result was found from MS + 10.0 mg/l BAP medium, 80% explant showed regeneration, 10 days required for initiation of shooting, mean number of multiple shoot 4, mean length of plantlet 3.0 cm, mean size of leaf is 1.0 cm and mean number of leaf were 11. Callus mediated regeneration of *Adhatoda vasica* shooting was not well reported by other researchers, most of them used callus for isolation of alkaloids was reported (Sunita M. and Dhananjay S., 2010).

4.4. *In vitro* initiation of rooting of *Adhatoda vasica*:

Rooting response of *in vitro* raised shoots is controlled by growth regulators. After shoot elongated, the healthy regenerated shoots from both nodal explants and callus, were rooted. For most of the species auxin is required to induce rooting. NAA and IBA are most commonly used for root induction (Bhojwani and Razdan, 1992). MS, ½ MS, MS + 1.0 mg/l IBA, ½ MS + 1.0 mg/l IBA & MS + 2.5 mg/l IBA + 0.5 mg/l IAA + 0.5 mg/l NAA were prepared for rooting. By the use of IBA many plants such as *Adhatoda vasica* (Raageeva bimal & Shahnawaz, 2012), *Lycopersicon esculentum* (Sibi, 1982), *Hedychium roxburgii* (Tripathi and Bitaillion, 1995) gave in vitro rooting. In the present investigation, MS + IBA (2.5 mg/l) + IAA (0.5 mg/l) + NAA (0.5 mg/l) performed best; root formation of explant was 80%, 12 days required for initiation of

rooting from shoot regenerated from nodal explants and root formation of explant was 60%, 13 days required for initiation of rooting from callus mediated shoot. By the use of IBA many plants such as *Adhatoda vasica* (Raageeva, B. & Shahnawaz, M., 2012), *Lycopersicon esculentum* (Sibi, 1982), *Hedychium roxburgii* (Tripathi and Bitallion, 1995) gave *in vitro* rooting.

4.5. Establishmemt of Plantlets to Field conditions:

In vitro propagated plantlets are not transferred readily to an open environment. A hardening stage is usually used in which the plants are transplanted from *in vitro* conditions in green house and then gradually keep them three to four weeks to that condition and then transferred to the open environment (Jones, 1983). In the present investigation, comparatively healthy rooted plants were taken out from the culture vessels and wash gently under running tap water to get rid of agar. Hardening of tissue culture plants is the most crucial step in micropropagation. The plants produced are very soft to face ambient environmental conditions (Bhojwani and Razdan, 1992). These plants are grown under controlled conditions. Under these conditions the leaves of plants develop cuticle and its photosynthetic system starts functioning. The most crucial stage is during first 3/4 days in polyhouse. During the second hardening stage, mortality is lower as the plants are comparatively hardened during first hardening stage or during the first 3/4 days covered with polybags under shade condition. After transplantation to garden soil most of the plantlets were established in field condition. The ultimate survival rate of the transferred plantlets to soil was about 80%. Same survival rate of acclimatized plantlets, was also reported (Raageeva, B. & Shahnawaz, M., 2012). The protocol described in this study is repeatable and long term *in vitro* regeneration of *Adhatoda vasica* plants using nodal explants and juvenile leaves. The protocol established in this study is also useful for conservation, commercial cultivation and genetic improvement of this medicinal plant.

Chapter five

Conclusion

5. Conclusion

In the present study, attempts were made to establish an efficacious procedure for large scale production of *Adhatoda vasica in vitro*. Multiple shoot regeneration and callus induction was done with nodal segments and juvenile leaves respectively as explants in this study. Multiple shoot formation, callus mediated shooting; rooting of *in vitro* raised shoots and establishment of plantlets to field conditions were experimented. MS media with various hormonal supplements namely, 2-4-D, IAA, NAA, IBA, BAP, GA_3, and Kn were used singly or in combinations for different purposes of *Adhatoda vasica* micropropagation.

For multiple shoot formation, MS medium supplemented with BAP (10.0 mg/l) was best in which 93.33% explants produced multiple shoots, 7 days required for initiation of shooting. The nodal explants remained green and viable. After 1 month, mean number of multiple shoot 10.6±1.82, mean length of plantlet 5.2±2.20 cm and mean number of leaf were 15.6±1.80.

Medium containing Kn (0.5 mg/l) + GA_3 (0.5 mg/l) + BAP (5.0 to 10.0 mg/l), IAA (0.5 mg/l) + NAA (0.5 mg/l) + BAP (5.0 to 10.0 mg/l) and IAA (0.5 mg/l) + Kn (0.5 mg/l)+ BAP (5.0 to 10.0 mg/l), showed poor response in shoot multiplication and some of these explants were no longer able to establish regeneration after subculture, were turned black or dried out.

In case of callus induction, maximum100% callus formation was observed on MS medium supplemented with IAA (0.05 mg/l) + NAA (0.05mg/l) + BAP (1.0mg/l) . 30 explants were inoculated; callus initiation started from 14 days, 30 explants showed callus formation and gave soft green coloured, fast growing callus. Media containing IBA (1.0 mg/l) with GA_3 (1.0 mg/l) also showed better response, 93.33% of responsive explants and callus gives light green colour.

GA_3 (1.0mg/l) with BAP (0.05 - 0.09 mg/l) combinations showed very poor response in callus induction, calluses were yellowish white to white, after few days these were turned brown in colour & are non viable.

Callus mediated shooting was found best from MS + BAP (10.0 mg/l) medium, 10 explants were transferred, 80% explant showed regeneration, 10 days required for initiation

of shooting, mean number of multiple shoot 4.0 ± 1.70, mean length of plantlet 3.0 ± 1.56 cm and mean number of leaf were 11.0 ± 1.45.

Best rooting response of *in vitro* raised shoots found on MS + IBA (2.5mg/l) + IAA (0.5 mg/l) + NAA (0.5 mg/l); root formation of explants was 80%, 12 days required for initiation of rooting from shoot regenerated from nodal explants and root formation of explant was 60% , 13 days required for initiation of rooting from callus mediated shoot.

Well rooted plantlets were transferred to plastic pots containing soil for hardening. After hardening, transplantation to garden soil was done. Most of the plantlets were established in field condition, the rate was about 78%.

Chapter six

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6. REFERENCES

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