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IN VITRO PROPAGATION OF *Angelonia angustifolia* BENTH .

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ABSTRACT

The present investigation entitled "*In vitro* **Propagation of Angeloina angutifolia Benth** belongs to the family **Plantanginaceae**. The present work was aimed towards the development of a protocol for the large scale multiplication of this important medicinal plant. **Murashige and Skoog (1962)** medium, was used as a basal medium with different concentrations and combinations of growth regulators. The explants, axillary buds and shoot tips were excised from the healthy mother plant selected for initiation of shoots. Fifteen different combinations were used for the induction and multiplication of shoots; among these MS-7 fortified with 2mg/L of BAP and 0.5mg/L of IAA was found to be the most suitable one for the initiation of shoots. Highest mean number of shoots (7.10±0.50) was observed on MS-7 fortified with BAP (2mg/L) + IAA (0.5mg/L) Healthy multiple shoots were produced on multiplication phase and these shoots can be taken over for the next stage of rooting.Twelve different combinations were used for the rooting of *in vitro* raised shoots; among these MS-17 fortified with 1mg/L of IAA was found to be the most suitable one for the initiation of shoots. In conclusion, we improved *in vitro* regeneration efficiency from axillary bud and shoot tip explants of *A.angustifolia* on MS medium supplemented with BAP alone and in combination with (IAA and NAA). Axillary bud explants protocol to be a better one for the adventitious shoot induction and produced more number of shoots when compared to shoot tip explants. Optimal concentration of BAP (2 mg/l) along with IAA (0.5 mg/l) increases the multiple shoot induction. This standardized protocol may be helpful for large scale propagation and also for the conservation of this important medicinal, ornamental species.

KEYWORDS: Angeloina angutifolia , 2-iso Pentenyl adenine, In vitro studied, Benzyl Amino Purine, EDTA

1. INTRODUCTION

Plants are one of the most important sources of medicines. Medicinal plants are of great significance to the human health including animal communities. The medicinal values of these plants are due to their some chemical compounds, which are called *phytochemicals* and they are non nutritive chemicals that has a protective or disease preventive property (Hill, 1952). Plant wealth is greatly exploited for its therapeutic potential and medicinal efficacy to cure various ointments from time immemorial. There is an increasing spotlight on the importance of medicinal plants and traditional health care systems in solving the health care problems of the world (Pal and Shukla., 2003).

Medicinal herbs/plants or the herbal drugs refer to the use of plant and plant-based products for the management of common ailments. World Health
Organization has defined herbal medicines as finished labeled medicinal product that contains an active ingredient, aerial, or underground parts of the plant or other plant material or combinations. According to the WHO, at present, over 80% of the world's population relies on traditional forms of medicine, largely plant based to meet primary health care needs (Winslow and Kroll).

In India, more than 70% of the population uses herbal medicine for their health-related problems. The ayurvedic, Siddha and Unani systems of medicines are widely used by the people of Indian subcontinent. Almost all civilization and cultures have employed plants in the treatment of human sickness (Samal, J., 2016). The Indian system of Ayurvedha is probably 5000 Years old which is based on the knowledge contained in Rigvedha and Atharvanavedha, the treatise charaka samhita (1900 BC) and Susruta Samhita (1500 BC). In India, the collection and processing of medicinal plants and plant products contributes a major part each year to the national economy, as a source of both full and part time employment (Holley et al., 1998).

As a result in the increase in the medicinal plant knowledge, the demand for the medicinal plant resources has been increasing due to the worldwide buoyancy in the herbal sector engaged in the production of herbal health care formulations; herbal based cosmetic products and herbal nutritional supplements. Thus, the international trade in plants of medical importance is growing phenomenally, often to the detriment of natural habitats and mother populations in the countries of origin. Due to the indiscriminate harvesting, the wild populations of many medicinal plant species, forming the major resource base for the herbal industry, are facing a serious threat of extinction (Rawat, 2008; Pandey et al., 2008; Soni, 2009).

Plant tissue culture has developed widely incorporated into biotechnology, the agricultural systems being a key factor to support many pharmaceutical and industrial outcomes. Since 1902 there is vast progress in plant culture and its application has emerged having a great diversity in the science filed. Due to development and desire to grow on high scale production in the past few decades, tissue culture techniques were manipulated for improvement of plant growth, biological activities, transformation, and secondary metabolites production. A significant advance in techniques has been sought to deal with problems of low concentrations of secondary metabolites in whole plants. The augmented use of plant culture is due to a superior perceptive of plant oriented compounds and secondary metabolites from economically important plants. Due to development in modern techniques, several particular protocols have been developed for the production of a wide array of secondary metabolites of plants on a commercial scale. Plant tissue culture has to lead to significant contributions in recent times and today they constitute an indispensable tool in the advancement of agricultural sciences and modern agriculture (Pierik, 1991).

Plant tissue culture refers to, as *in vitro*, to growing and multiplication of cells, tissues and organs of plants on defined solid or liquid media under aseptic and controlled environment. The commercial technology is primarily based on micro propagation, in which rapid proliferation is achieved from in system cuttings, axillary buds, and to a limited extent from somatic embryos, cell clumps in suspension cultures and bioreactors (Hameed, *et al*., 2006).

Plant tissue culture is a method to culture the cells, tissue organs and other components of the plant following the aseptic in-vitro culture under a well-defined environment. In a simple way, if a part of the plant body is dissected into a small part which is called explant and that can be grown into a complete plant. The explant exhibits a very high degree of plasticity in-vitro, thereby allow the explant to develop into another type and this way a whole new plant can be subsequently regenerated. To grow a full fledge plant; any portion of plant can be grown into explants using the growth media (Krikorian and Berquam, 1969).

Tissue culture is a proven means of producing millions of genetically uniform plants under aseptic condition. It is not only reducing the time and space but also gives greater output and allows further augmentation of elite disease free plants. The commercial production of these crops is restricted due to the shortage of desirable planting material or due to various other factors like urban development, deforestation, and indiscriminate collection of plants (Bhojwani and Razdan, 1986).

The main objectives of this study are

- To develop a protocol for the mass propagation of selected plant through tissue culture.
- To standardise concentration of the plant growth regulators for the micro propagation of the selected species.

With these objectives, the present study has been carried out on *in vitro* morphogenetic studies of a common oranamental and medicinal plant, *Ochna serrulta* belonging to the family Ochnaceae.

2. REVIEW OF LITERATURE

Medicinal and aromatic plants play a significant role in the life of people and are present in innumerable forms. In Indian traditions, all the plants in this earth are considered as medicinal (Jivak in Astanga Hriday (Sutra: 9-10)). However, a simplest definition of the medicinal plant would be "Medicinal plants are those plants which are used in official and various traditional systems of medicines throughout the world". Other definition could be "Medicinal plants are plants that provide people with medicines - to prevent disease, maintain health or cure ailments". In one form or another, they benefit virtually everyone on earth. Still, no exact definition of Medicinal Plant is possible. There are related issues, such as for nutrition, toiletry, body care, incense and ritual healing.

India has been considered as treasure house of a large number of valuable medicinal and aromatic plant species. Ministry of Environment and Forests, Government of India has identified and documented over 9500 plant species considering their importance in the pharmaceutical industry. Over 90% of the medicinal plants traded in India are harvested from the wild, most of them in an unsustainable manner (WWF- India and FRLHT, 2007). Due to an increasing demand for medicinal plants and a loss and fragmentation of natural habitats, close to 366 species of Indian medicinal plants have been so far assessed as under threat in the wild (based on International Union for Conservation of Nature (IUCN) Red List Criteria) (Vie et al., 2009). Around 1,000 species are estimated to be facing various degrees of threat across different bio-geographic regions in the country. It is, therefore, relevant that these valuable plant species are not only preserved but also their cultivation practices are developed in order to meet the demand. Shift from collection to cultivation of medicinal plants will also ensure purity, authenticity and sustainable supply of raw drugs.

2.1 *Angelonia angustifolia BENTH*.

The *Angelonia angustifolia* benth (1812) is usually erect perennial herbaceous species .30-80cm tall ,with pubescent quadrangular stems, simple or ramified at the base , and opposite sessile leaves ,decussate,

simple, oblong with ponitedapex and slightly toothed margins , 3-10 cm long and 5-18 cm broad , of decreasing dimensions towards the apex , covered by tiny glandular hairs on both faces , aromatic. Solitary flowers at the axil of the terminal leaves , on an about 1-cm long peduncle 2-4cm broad with green calyx with 5lobes , about 4cm long, ovate-lanceolate with pointed apex and provided of glandular hairs . Almost bilaiate corolla, of more or less violet colour,with concave throat , biparted upper labrum and triparted lower one with the intermediate part longer, and round margins , 4 stamina and pubescent spherical ovary.The fruit is abilocular globose capsule containing numerous seeds. Plant tissue culture techniques provide a unique approach for conservation and mass propagation for any economically important plant species. There is only one report on the tissue culture studies in this plant. Due to their immense potential applications for wellbeing of making, a successful attempt has been made towards development of an efficient mass propagation protocol for *A.angustifolia* using tissue culture techniques. Systematic position

Kingdom Plantae – Plants

Subkingdom Viridiplantae – Green plants

Superdivision Embryophyta – land plants

Division Tracheoiophyta –vascular plants

Class Magnoliopsida – Dicotyledons

Super order asteranae

Order Lamiales

Family Plantaginaceae – Plantains family

Genus *Angelonia BENTH*.

Species *Angelonia angustifolia*. – Narrowleaf angelon

Taxon – *Angeloina angustifolia BENTH*

Synonyms:

Angelonia acuminatissima Herzong

Angelonia alternifolia V.C. Souza

Angelonia argute Benth.

Angelonia biflora Benth.

Angelonia hirta Cham.

Angelonia salicariifolia BONpl.

Angelonia verticillata Philcox

Vernacular Names

Willowleaf Angelonia, Summer snapdragon, Angel flower, Purple pasion

Etymology

Most Angelonia species can be found in Northeastern Brazil in the seasonally Dry Tropical forest namely caatinga. The flowers of Angelonia are highly specialized for pollination because they have hairs in the inner corolla, which produces oils collected by oil bee pollinators

Distinguishing Features

A small woody shrub with rough hairy-textured bark.

Its alternately arranged leaves are narrowly oval or elongated and have finely toothed margins.

Its bright purple flowers (2-3 cm across) have five petals.

4 stamina and pubescent spherical ovary.

The fruit is abilocular globose capsule containing numerous seeds.

Distribution

Angelonia angustifolia is a native to mexio, Cental America and Tropical South America.

Ecology

The flower of Angeloina are highly specialized for pollination because they have hairs in the inner corolla, which produces oils collected by oil bee pollinators,especially of the genus Centris.

2.2. Plant cell, tissue and organ culture

With the continued expansion of in vitro technologies, it is now possible to culture plant cells in a variety of ways: individually (as single cells in microculture systems); collectively (as calluses or suspensions, on Petri dishes, in Erlenmeyer flasks, or in large-scale fermenters); or as organized units, whether it is shoots, roots, ovules, flowers, fruits, and so forth. In simple terms, plant-cell culture can be considered to involve three phases: first, the isolation of the plant (tissue) from its usual environment; second, the use of aseptic techniques to obtain clean material free of the usual bacterial, fungal, viral, and even algal contaminants, and third, the culture and maintenance of this material in vitro in a strictly controlled physical and chemical

environment. An extra, fourth phase may also be considered where recovery of whole plants for rooting and transfer to soil is the ultimate goal (Hall, 1999).

The term "Tissue culture technique" colloquially covers a wide range of techniques including ***in vitro*** culture of organs (shoot tips, root tips, flowers, ovaries, ovules, and embryo's anthers etc.), tissues, cells and protoplasts. The role of medicinal plants and species can be visualized under the following categories

1. Germplasm conservation.
2. Freeze preservation.
3. Mineral growth.
4. Rapid clonal propagation.
5. Production of virus – free Plants.
6. Production of pharmaceuticals and other constituents.
7. Genetic improvement.
8. Isolation of mutants.
9. Production of haploids
10. Protoplast fusion and somatic hybridization.
11. Culture of immature hybrid embryos.

2.2.1. Totipotency

The theoretical framework and experimental basis of modem plant biotechnology derive from the concepts of cellular totipotency (the ability of a single cell to divide and produce a whole plant) and genetic transformation (genetic alteration caused by the uptake, stable integration and expression of foreign genetic material). The concept of totipotency is inherent in the Cell Theory of Schleiden (1838) and Schwann (1839), which recognized the cell as the primary unit elementary part of all living organisms. Totipotency implies that all the information necessary for growth and reproduction of the organism is contained in the cell. Although theoretically all plant cells are totipotent the meristematic cells are best able to express it. The Austro-German botanist Gottlieb Haberlandt was the first to try to obtain experimental evidence of totipotency by culturing plant cells in nutrient solutions in the hope of regenerating whole plants (Vasil, 2008).

The process of specializing cells' functions is called cell differentiation. It is accompanied by morphogenesis, the change of the cells' morphology.
Differentiation is done by turning on certain genes and turning off some others at a certain time. Therefore, for a highly differentiated cell to grow into a full plant, the differentiation process has to be reversed (called

de-differentiation) and repeated again (called re-differentiation). These two phenomena of dedifferentiation and redifferentiation are inherent in the capacity described as cellular totipotency, a property found only in plant cells. Theoretically-, all living cells can revert to an undifferential status through this process. However, the more differentiated a cell has been, the more difficult it will be to induce its de-differentiation. Practically, the younger or the less differentiated a cell is, the easier to culture it into a full plant. The ease of fulfilling the cell totipotency also varies tissue by tissue, genotype by genotype and species by species. Genotype dependency is often the bottle-neck in plant tissue culture and also in plant genetic engineering (Narayanaswamy, 1994;

Srivastava, 2002;

http://croptechnology.unl.edu/printLesson.cgi?lessoniD=956783940). All these including plant cell, tissue and organ culture have potential application in various areas (**Fig.1**).

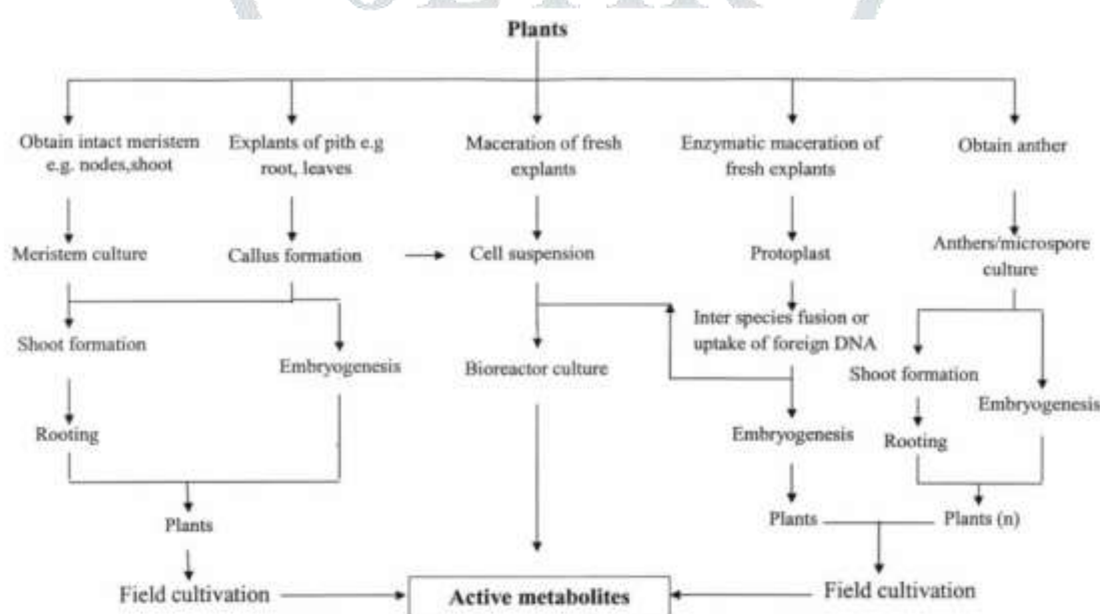


Figure 1. Schematic diagram of the major areas of plant cell, tissue and organ cultures and some fields of application

Source: Neumann *et al.*, (2009)

2.2.2. Micropropagation

The major invention of tissue culture came after the discovery of the cytokinin, in 1956 (Skoog and Miller, 1957). Since their discovery, cytokinins have been implicated to play a role in almost all aspects of plant growth and development, including cell division, shoot initiation and growth, leaf senescence, and photomorphogenic development (Mok and Mok, 1994). Micropropagation strategies have been developed for a number of economically important and threatened plant species and more and more species are becoming amenable for micropropagation (Rout et al., 1997). In micropropagation, buds from a desired plant are placed

on an appropriate culture medium under specific growth conditions to enhance production of auxiliary shoots. Sub culture of the buds and shoots is repeated until many plants are produced all having the genetic characteristics of the original plant (Hussey, 1983).

In vitro propagation also provides a means of germplasm storage for maintenance of disease free stocks (Wilkins and Dodds, 1983; Withers, 1989). The major routes presently used for regeneration of plants in in vitro conditions are (i) Culture establishment, (ii) Shoot development and multiplication and (iii) Rooting and hardening of plantlets. The optimum requirements for each stage must be empirically determined, although the broad principles to be used have been established (Gangopadhyay et al., 2002; Sanjaya et al., 2006; Sharma et al., 2009;

Balaraju et al., 2009).

2.2.3. Shoot development and multiplication

The media formulation (MS medium) described by Murashige and Skoog (1962) is the most commonly used. Basically, a nutrient medium consists of all the essential major and minor plant nutrient elements, vitamins and a carbohydrate as carbon source with other organic substances as optional additives. The composition of different nutrient media and the nature, source and use of the ingredients have been discussed in detail by Murashige (1974) and Torres (1989). The levels and kinds of plant growth regulators included in the culture medium largely determine the success of tissue culture. Shoot and root initiation, and the process of differentiation from unorganized callus tissue, are closely regulated by the relative concentrations of auxins and cytokinin in the medium (Ammirato, 1983; Bajaj et al., 1988; Rout and Das, 1997). Auxin: cytokinin ratios of ~ 10 yield rapid growth of undifferentiated callus, a ratio of ~ 100 favors root development and a ratio of ~ 4 favors the development of shoots (Murashige, 1980).

2.2.4. Rooting of in vitro raised plantlets

There is marked variation in the rooting potential of different plant species, and systematic trials are often needed to define the conditions required for root induction. In vitro induction of roots from growing shoots has been achieved in standard media with and without auxins depending on plant genotype (Rout et al., 1989). Moderate to high concentrations of all cytokinins are reported to inhibit rooting. Rooting has been reported to improve in many woody and herbaceous species when the concentration of macro salts were lowered to half or less, and the concentration of sucrose were lowered from 2 or 3% to 0.5% (Webb and Street,

1977). In vitro rooting of Asclepiadaceae members have been reported by Reddy et al., (1998), Koma1avalli and Rao (2000), Beena et al., (2003), Martin (2002) and

Faisal et al., (2007).

2.2.5. Acclimatization

The benefit of any micropropagation system can only be fully realized by the successful transfer of plantlets from tissue-culture vessels to the ambient conditions found ex vitro. Most species grown in vitro require an acclimatization process in order to ensure that sufficient number of plants survive and grow vigorously when transferred to soil. Since a substantial number of micropropagated plants do not survive transfer from in vitro conditions to greenhouse or field environment, the transplantation stage continues to be a major bottleneck in the micropropagation of many plants. This has been attributed to the external conditions of the greenhouse and field which have substantially lower relative humidity, higher light level and septic environment that are stressful to micropropagated plants compared to in vitro conditions (Hazarika, 2003). Acclimatized plants belonging to Asclepiadaceae have been reported earlier. Reddy et al., (1998) reported acclimatization of in vitro raised

G. sylvestre plantlets to tubes containing autoclaved tap water initially and after 810 days transfer the same to plastic pots (9 x 9 em) containing au toe laved soilrite covered with perforated polythene bags under culture room conditions and maintain humidity for 7 days to obtain a transplantation success of 75%. Komalavalli and Rao (2000) reported acclimatization of in vitro rooted *G. sylvestre* plantlets (5-6 cm height) with 80-85% success. *Ceropegia candelabrum* plantlets were acclimatized using sterile soilrite + sand in 1:1 ratio with 90% survival within 15 days (Beena et al., 2003). Martin (2004) acclimatized *Leptadenia reticulate* plantlets in small pots containing sterile sand and soil (1: 1) with 92% of survival within 15 days. Vermiculite has been used to acclimatize in vitro raised *Tylophora indica* plantlets with highest survival rates (Faisal et al., 2007).

2.3. Field establishment

Cultivation of some herbs has proved difficult because of low germination rates or specific ecological requirements. Controlling the growing environment makes it feasible to control phenotypic variation in the concentration of medicinally important compounds present at harvest. The control of light, temperature, water availability and soil composition can all therefore feasibly be manipulated to achieve the desired effects at harvest (Canter *et al*., 2005). Altering the environmental conditions has been reported to reduce alkaloid

content in *Atropa belladonna*, reduce essential oil content and menthol content in *Mentha piperata*, increase morphine content but reduce alkaloid content in *Papaver somniferum* (poppy) which are reported to be affected by climatic conditions, water availability, exposure to soil microorganisms and variations in soil pH and nutrients (McChesney, 1999). Though awareness on cultivation of G. sylvestre came in late 1990s, still studies are limited. Subbaraj and Thamburaj (1997) found that vines of G. sylvestre needed a fertilizer dose of 10 kg N + 2.5 g per vine for better growth of plants. Prasad et al., (2002) reported that application of 17:17:17 NPK ratio at 125 kg/ha to encourage maximum biomass production (yield), number of primary and secondary branches, leaf area, and fresh and dry weight. Karikalan et al., (2002) while conducting an intercropping experiment to study the nitrogen management on The growth and yield of *G. sylvestre* in a kapok (*Ceiba pentandra*) agroforestry field and an open field reported better plant height and higher number of leaves in the intercropping field. The production of biomass and evaluation active metabolites in the established field has not been reported. As the study was looks for further development in the field.

2.4. *In vitro* production of *A.angustifolia BENTH.*

A micropropagation protocol was for developed for *A.angustifolia* using axillary shoot buds by Goel et al., (2008). Goet et al., (2008) found of all the cytokinis i.e. BAP, Kinetin and Methyl amino purine, BAP was found most suitable for shoot multiplication followed by kinetin and MAP. Rapid in vitro shoot multiplication was most optimum on basal MS medium supplemented with BAP (2.0 mg/L) and IBA (0.25 mg/L). The shoots were successfully rooted on half strength semi-solid basal MS medium with 2% sucrose containing IBA (1.0 mg/L).

Table -1. The other in vitro studies in medicinal plants are below

Bothanical name	Therapeutic use	Explant used	Response	Reference
Adhatoda Vasica	Antibronchitis	Stem Segment	Plantlets	Jaiswal *et al.,* (1989)
Ammi majus	Dermatological diseases	Seedling	Plantlets with flowers	Purohit *et al.,* (1995)
Artemisia annua	Antimalarial	Nodal segment	Plants lets	Gulati *et al.,* (1996)
Atropa belladooa	Spasmolytic	Shoot tip	Multiple Shoots	Gulati (1987) Shekhawat *et al.,* (1995)
Azadirachta indica	Antiseptic	Shoot tip	Shoots	Ramesh & Pandhya (1990)
Allium Sativum	Antihelmintic	Meristem tip	Shoots , roots	Novak *et al.,* (1982)
Bacopa Monniera	Memory Vitalizer	Nodal segments	Plantlets	Ali *et al.,* (1996)

Butea monosperma	Antihepatotoxic	Cotyledons	Plantlets	Mughal *et al.,* (un published)
Boerhaavia diffusa	Diuretic	Shoot lips	Shoot buds	Sudharsana Santhamma (1988)
Catheranthus roseus	Antileukamic	Stem segments	Plants lets	Datta & Srivatsava
Capsicum annum	Carminative	Cotyledon	Plants lets	Christoper *et al.,* (1991) Shekhawat *et al.,* (1995)
Coccinia grandis	Antidiabetic	Shoot tip	Shoot buds	Gulati *et al.,* (1988) Shekhawat *et al.,* (1995)
Cinchona ledgeriana	Antimalarial	Seedlings	Shoots	Hunter (1998)
Coptis japonica	Expectorant, Anti helmintic	Pedicel	Plantlets	Nakagawa *et al.,* (1982)
Crataeva nuruala	Expectorant, Antihelmintic	shoot tip	Embryoids	Inamder *et al.,* (1990), Shekhawat *et al.,* (1995)
Coriandrum sativum	Carminative heart tonic	Seedling	Embryoids	Schafer *et al.,* (1986)
Datura innoxica	Antispasmolytic	Anther	Plantlets	Srivatsava *et al.,* (1993)
Datura metal	Antispasmolytic	Anther	Embroyogene sis	Babber & Gupta (1990) Shekhawat *et al.,* (1993)
Digitalis lanta	Cardiotonic	Shoottip	Plantlets	Luckner *et al.,* (1984)
Delonix regia	Agglutinamation	Leaves	Plantlets	Gupta *et al.,* (1996)
Dioscorea deltoidea	Preursor of Glucocortico Steroids	Shoot tips	Embryoids	Sharma & Chathurvedi *et al.,* (1989)
Duboisia myoporoides	Sedative emetic	Nodal Segments	Plantlets	Kukreja & Mathur (1985)
Gentiana cruciaia	Emmenogogue	Embryos	Plantlets	Weslowska *et al.,* (1985)
Glycyrrhiza glabra	Antidiabetic	Embryos	Plantlets	Shah & Dalall (1982)
Heliotropium indicum	Anticancerous	Seedling	Plantlets with flowers	Data (1996)
Hyoscyamus niger	Antispasmolytic	Seedling	Plantlets	Cheng & Raghavan (1985)
Lepidium Sativum	Antibacterial	Cotyledonary leaf	Plantlets	Pande (1996)
Moringa Oleifera	Anti viral	Hypocotyl	Plant lets	Mohan *et al.,* (1996)
Panax ginseng	Tonic	Anther	Plantlets	Du *et al.,* (1986)
Papaver Somniferum	Analgesic	Hypocotyl	Plantlets	Nesslar (1980)
Picrorhiza kurroa	Cardiotonic	Shoot tips	Plantlets	Lal etal (1988)

				Shekhawat *et al.*,(1995)
Podophyllum hexandrum	Anticancerous	Embryo	Embryoids	Arumugan (1989)
Plantago Ovata	Laxative	Cotyledon	Plantlets	Wakhul Barna (1989)
Rauwolfia serpentina	Hypertensive sedative	Various parts of Plants	Plantlets	Shekhawat *et al.*, & Chaturvedi *et al.*, (1982)
Riccinus communis	Lubricant purgative	Shoot, root , cotyledon	Callus , Plantlets	Anthma & Reddy (1983)
Santalum album	Diuretic expectorant	Nodal segment	Plantlets	Lakshmi sita (1986)
Silybum marianum	Anti hepatotoxic	Nodal Segment	Plantlets	Saba *et al.*, (1997)
Syzygium cumini	Antidiabetic	Stem	Plantlets	Yadav *et al.*, (1990) Shekhawat *et al.*,
Syzygium aromaticum	Carminative	Stem	Shoots, Plantlets	Jadish, Chandara & Ravishankar rai (1986)
Taraxacum officinale	Hepatic, Stimulant Diuretic	Root	Shoots	Bowes (1970)
Taxus baccata	Anticancerous	Young shoot cuttings	Callus	Nandi *et al.*, (1997)
Thevetia periviana	Heart diseases	Stem segments	Plantlets	Gulati (1997)
Thymus vulgaris	Antifungal, antihelmintic	Shoottip	Plantlets	Oiszowske (1982)
Tilophoro fienum, graecum	Antidiabetic	Mesophyll Protoplast	Shoot	Shekhawat & Galston (1983)
Tylophoro indica	Antiasthmatic emetic	Stem segments	Shoot segments	Mhatra *et al.*, (1984), Shekhawat *et al.*,
Urginea indica	Cardiotonic	Bulb Scale	Callus, Plantlets	Jha *et al.*, (1994) Shekhawat et al.,
Urginea maritime	Cardiotonic expectorant	Leaf	Plantlets	Dohnal *et al.*, (1986)
Vinca minor	Bleeding piles Antidysentric	Shoot lip & Nodal segment	Shoot, Plantlets	Stapfer &Houser (1985)
Withania Somnifera	Antispasmodic	Anther	Plantlets	Vishnoil *et al.*, (1979)
Zingiber officinale	Carminative digestive stimulant	Stem segment	Plantlets	Balachandar *et al.*, (1990) Shekhawat *et al.*, (1995)

III. MATERIALS AND METHODS

General:

General laboratory techniques recommended by Purivis *et al.,* (1966) and Dodds and Rpberts (1984) were followed for sterilization, preparation of media, inoculation and maintenance of cultures.

Cleaning solution :

Cleaning solution is prepared as proposed by Mahadevan and Sridhar, (1996) it contains,

Potassium dichromate	- 60 g
Concentrated H_2SO_4	- 60 ml
Distilled water	- 1000 ml

Potassium dichromate was dissolved in warm water and cooled. To which concentrated sulphuric acid was added slowly and mixed thoroughly and made up to 1000 ml with distilled water and solution was used for cleaning glass wares.

Cleaning of Glassware's:

All glassware's were cleaned thoroughly before conducting experiments. New glass wares were washed with water and detergent [preferably liquid detergent like, Teepol, Vim liquid, Labolin, etc.,] followed by thorough rinsing with tapwater and then with distilled water. Used glassware containing deposits, were immersed in a cleaning solution. After removing from the cleaning solution, the glassware were thoroughly cleaned with water and detergent and finally rinsed with water.

Sterilization :

The glasswares includes petridishes, pipettes etc., may be sterilized by placing them in hot air oven at 180 °C for two hours. In case of culture media, blade holder, forceps, tweezers and scissors were sterilized in an autoclave at 121°C for

15-20 minutes at 15 psi of pressure.

Chemicals :

All analytic grade chemicals were used and procured from Merck (India), SRL (India) and Hi-Media Mumbai (India) and Sigma (USA) for preparation of tissue culture media, stock solutions and reagents.

Nutrient Medium :

Growth and morphogenesis of plant tissues ***in vitro*** are largely governed by the composition of the culture media. For the present investigation Murashige and Skoog (1962) basal medium was used. The media composition of Murashige and

Skoog (1962) is shown in the **Table – 2.**

Table – 2. Composition and Stock Solution of Murashige and Skoog medium (1962)

Composition	Stock	Constituents	Conc.of Stock (g/l) Solution	Vol.of stock to be Taken (ml/l)	Final Conc in one litre medium (mg/l)
Macro Salts (100x)	A	KNO_3	190	10	1900
	B	NH_4NO_3	165	10	1650
	C	$CaCl_2H_2O$	44	10	440
	D	$M_gSO_4.7H_2O$	37	10	370
		KH2PO4	17		170
MicroSalts (200x)	E	$M_nSO_4.4H_2O$	4.46	5	2.23
		$ZnSO_4.7H_2O$	1.72		8.6
		H3BO3	1.24		6.2
	F	KI	0.166	5	0.83
		$Na_2M_0O_4.2H_2O$	0.05		0.25
	G	$CuSO_4.5H_2O$	0.005	5	0.025
		$CoCl_2.6H_2O$	0.005		0.025
Iron Source 200x	H	Na_2. EDTA	7.46	5	37.3
		$FeSO_4$. $7H_2O$	5.56		27.8
Vitamins 200x	I	Thiamine HCl	0.1	5	0.5
		Nicotinic Acid	0.1		0.5
		Pyridoxine HCl	0.1		0.5
	J *	Myo – Inositol	-		100
Amino Acid	K	Glycine	0.4	5	2.0
Carbon Source	L *	Sucrose	-	-	30 g/L
Gelling Agent	M*	Agar	-	-	8 g/L

Note: * Added Freshly.

Preparation of Stock Solutions :-

Stock solution of macro and micro nutrients, vitamins, iron source, amino acid and plant growth regulators were prepared in double distilled water. The stock solution of macro nutrients was prepared as 100x concentration. The stock solution of micro nutrients, Iron source, vitamins and amino acids were prepared as 200 x concentration as shown in the **Table – 2**. Iron stock solution was prepared by dissolving $FeSO_4.7H_2O$ and $Na_2EDTA.H_2O$ separately in 450 ml of double distilled water by heating with constant stirring. Mix the

two solution and adjust the pH to 5.8 and added double distilled water to make up the final volume to one liter. Iron stock solution was stored in amber bottle to prevent photolysis. All the stock solutions were stored in refrigerator and myo-ionisitol, growth regulators and sucrose were freshly prepared and used.

Plant Growth Regulators:

The following plant growth regulators were used, **Auxins :**

- 2,4, -D – 2,4-Dichlorophenoxy acetic acid
- IAA – Indole -3- acetic acid
- IBA – Indole -3- butyric acid
- NAA- Naphthalene acetic acid

Cytokinins

- BAP – 6 - Benzyl amino purine (or) 6-Benzyl Adenine
- Kn – Kinetin
- Ads – Adenine sulphate

Preparation of Auxins

The auxins namely 2, 4-D, IAA, IBA and NAA were prepared separately by dissolving 100mg in 2ml of 1N Sodium hydroxide and the volume was then made up to 100ml in a standard flask by addition of double distilled water and stored at 4°C, in the case of IAA it was stored in amber bottle (or) bottle covered with a black paper and kept in dark since they are unstable in light.

Preparation of Cytokinins

The cytokinin namely 6-Benzyl amino purine and kinetin (Kn) were prepared separately by dissolving 100mg in 2ml of IN sodium hydroxide and the volume was the made up to 100 ml in standard flask by addition of double distilled water and stored at 4°C, in case of kinetin was stored in amber bottle and kept in dark since they are unstable in light. Ads is added as fresh addendum

Preparation of culture medium

For preparation of medium, 1000ml beaker was taken with ¼th of sterile distilled water and added 3% of sucrose (30g/L), 100mg of myo-inositol stirr vigorously until completely dissolved. To which stock solution were added one by one as volume of stock to be taken (ml/L) shown in the **Table – 2**. The medium was mixed thoroughly and added required amount of plant growth regulators (PGRs) **(Table – 3 and 4)** and made up to one litre by addition of sterile distilled water. The pH was adjusted to 5.8 with 1 N HCl (or) 1N NaOH. The gelling agent Agar 0.8% (8 g/L) was added to the prepared media constituents by heating (100°C) the medium

until the medium become transparent or crystal clear which show that agar was completely dissolved in the medium. Then the media constituents were dispensed in to the culture tube of required volume (20ml). The content were labeled and sterilized in an autoclave at 15 psi of pressure for 15 min at 121 °C of temperature after autoclaving the culture media tubes were placed on a slanting stand or vertical stand and kept inside the culture room for incubation.

Source of plant

The healthy plant of ***Angeloina angustifolia*** was grown in Pachaiyappa's College Chennai – 30 (**Fig.2a; 2b**). Explants such as shoot tip, nodal segments were taken from these plants for all investigation.

Preparation of Explants

The explants were excised from the healthy mother plants which are

maintained in the College (**Fig. 2c**). The explants namely leaf, nodal segments, and shoot tips were washed thoroughly in running tap water overnight and then add few drops of soap solution like teepol, twin 20 etc., and washed thoroughly in running tap water and wash with 70% ethanol.

The washed explants transferred in to laminar Air flow chamber where the surface sterilization was carried out with the help of surface sterilizing agent mercuric chloride ($HgCl_2$).

Sterlization of Explant

The explants were collected from healthy plants maintained in green house. The explants such as nodal segments, shoot tips were selected to study the morphogenetic response. They were washed thoroughly in running tap water for 10 min. And then it was washed by using Tween – 20 for 10 min. These were washed thoroughly with running tap water. After the washing, the explants were subjected for different concentrations of mercuric chloride. Three different concentrations viz; 0.05, 0.1, 0.15 for 8 minutes were used to standardize the surface sterilization of various explants. The inoculations of explants were carried out under aseptic condition in Laminar flow chamber. The UV light in the chamber was switched on for an hour prior to use.

Inoculation

Inoculation was done under the Laminar Air flow chamber. The work area of LAF was thoroughly wiped with 80% alcohol and UV lamp keep switched on for 30 minutes to kill or denature the microbes before sterilizing function. The inoculation processes were carried out with the help of sterilized forceps and scalpel. The explants were sized to 0.5 cm – 1.0 cm length and placed on culture medium. Before going to each and

every operation forceps and knife were flame heat sterilized with the help of spirit lamp. After inoculation the culture tube were closed tightly by sterilized cotton plugs and allowed for incubation.

Incubation

Inoculated culture tubes were incubated in the culture room, where essential incubation parameters are maintained. The incubation parameters are as follows;

Temperature - 25 ± 2°C

Light intensity - 2000 – 4000 Lux

Photo period - 16/8 hr [Light/ Dark]

Table – 3. Various concentration and combinations of plant growth regulators for shoot proliferation.

	PGR in (mg/l)		
Basal Medium	**BAP**	**IAA**	**NAA**
MS-1	1	-	-
MS-2	2	-	-
MS-3	3	-	-
MS-4	4	-	-
MS-5	5	-	-
MS-6	1	0.5	-
MS-7	2	0.5	-
MS-8	3	0.5	-
MS-9	4	0.5	-
MS-10	5	0.5	-
MS-11	1	-	0.5
MS-12	2	-	0.5
MS-13	3	-	0.5
MS-14	4	-	0.5
MS-15	5	-	0.5

Table – 4. Various concentration and combinations of plant growth regulators for root proliferation.

PGR in (mg/l)			
Basal Medium	**IAA**	**IBA**	**NAA**
MS-16	0.5	-	-
MS-17	1.0	-	-
MS-18	1.5	-	-
MS-19	2.0	-	-
MS-20	-	0.5	-
MS-21	-	1.0	-
MS-22	-	1.5	
MS-23	-	2.0	-
MS-24	-	-	0.5
MS-25	-	-	1.0
MS-26	-	-	1.5
MS-27	-	-	2.0

Subculture & Maintenance

All the cultures were incubated for 4 – 5 weeks. After that the cultures were taken for next stage in the fresh media.

To study the morphogenetic potential of the culture at 15 replicates were maintained for each individual treatment.

4. OBSERVATIONS AND RESULTS

The axillary bud and shoot tip explants were collected from the healthy plants of *Angeolina angustifolia* for *in vitro* micropropagation study (**Fig: 2c**). The explants were collected, surface sterilized and inoculated on Murashige and Skoog (1962) medium supplemented with various concentration and combinations of plant growth regulators in order to obtain shoot induction and multiplication. After inoculation the cultures were incubated under the aseptic condition and the results were recorded by the periodical observations.

Induction of Shoots

The axillary buds and shoot tips of *Angelonia angustifolia* were used as explant for direct regeneration (**Fig: 2c**). The explants were inoculated on MS medium supplemented with various concentrations of BAP, IAA and NAA. The responses towards the induction of shoots were observed periodically and the results were tabulated in (**Table - 5**). All the concentrations of PGRs supplemented with MS medium were responded towards the shoot induction. The percentage of shoot inductions was varied.

Multiple shoot production from nodal explants

The production of plants from axillary buds has proved to be the most applicable and reliable method of *in vitro* propagation. For surface sterilization, mercuric chloride ($Hg\ Cl_2$) was used to standardize the healthy cultures. Nodal and leaf explants were surface sterilized with $Hg\ Cl_2$ (0.1 % w/v) for 5min and (0.5% w/v) for 4 min.

Different experiments were conducted to find an optimum culture condition for maximum shoot multiplication from the cultured explants. Nodal explants cultured on MS medium with various concentration of BAP (1-5 mg/l) showed shoot regeneration after 15 days of incubation without any callus formation. More multiple shoots were observed from the nodal explants cultured on MS medium supplemented with BAP (1-5 mg/l) alone or in combination with NAA (0.5 mg/l) and IAA (0.5 mg/l).

The medium supplemented with BAP alone showed poor shoot induction (34 no of shoots) per explants. There was no sign of shoot induction when explants were cultured in the media without any plant growth regulators. Among the several combinations tested, the media combination with BAP (2 mg/l) + IAA 0.5mg/l) found to be the most effective combination towards the production of multiple shoots (**Fig. 2e; 2f**). It was observed that incorporation of auxin in the medium at all levels of BAP improved the multiple shoot induction.

Initiation of multiple shoots was observed within six weeks of culture in most of the treatments. The maximum response of shoot induction was observed on MS medium supplemented with BAP (2 mg/l) and IAA (0.5mg/l) which produced 7.1±0.5 number of shoots with an average length of 4.2 ±0.2cm. (**Table - 5**). The medium supplemented with 2 mg/l of BAP alone recorded 60% response but produced more number of shoots (4.0) per explants with the shoot length of 3.0 cm (**Fig. 2f**). When NAA and IAA were applied alone the response was very poor in all the concentrations employed. At the end of the experiment, percentage of shooting, shoot length and the number of shoots per explants were recorded. After six weeks of incubation the well grown cultures were selected for root induction.

Table - 5. Effect of BAP, IAA and NAA on shoot proliferation from Nodal explants of *A.angustifolia* on MS medium

Basal Medium	PGR in (mg/l)			% of response	No of shoots/ explants	Shoot length in cm
	BAP	IAA	NAA			
MS-1	1	-	-	50	2.6±0.00	1.6±0.00
MS-2	2	-	-	60	4.0± 0.00	3.1±0.48
MS-3	3	-	-	45	3.0± 0.53	3.5±0.62
MS-4	4	-	-	40	2.1± 0.16	2.7±0.06
MS-5	5	-	-	30	2.0 ± 0.00	1.2±0.45
MS-6	1	0.5	-	60	3.0± 0.2	2.0±0.33
MS-7	**2**	**0.5**	**-**	**80**	**7.1± 0.50**	**4.2±0.20**
MS-8	3	0.5	-	40	5.1± 0.23	3.6±0.41
MS-9	4	0.5	-	35	3.0± 0.20	3.3±0.20
MS-10	5	0.5	-	20	1.8± 0.15	2.5±0.22
MS-11	1	-	0.5	35	2.6± 0.17	1.6±0.25
MS-12	2	-	0.5	25	2.0± 0.50	1.2±0.85
MS-13	3	-	0.5	35	2.3± 0.30	3.1±0.20
MS-14	4	-	0.5	40	2.6± 0.00	1.6±0.00
MS-15	5	-	0.5	45	3.0± 0.20	3.3±0.20

There were 6 explants in each treatment and data (* ± SD) were recorded after six weeks culture.

Rooting

Half strength MS medium supplemented with IBA (0.5-2.0mg/l), NAA (0.52.0mg/l) and IAA (0.5-2.0mg/l) was most effective in inducing roots. Among the various auxin concentrations tried 1 mg/l of IAA was found as suitable concentration towards the root induction in which 80 percent of the shoots produced quality roots within 21 days of incubation (**Fig. 2g & Table - 6**). This medium facilitated quality root induction with 4.6 ± 0.32 number of roots with an average length of 5.5±0.92 cm.

Table - 6. Effect of auxins on rooting from *in vitro* raised shoots ofA.angustifolia

Basal Medium	PGR in (mg/l)			% of response	No of roots/ explants	Roots length in cm
	IAA	IBA	NAA			
MS-16	0.5	-	-	42	3.2±0.32	2.2±.0.21
MS-17	**1.0**	**-**	**-**	**80**	**4.60±0.32**	**3.5±0.92**
MS-18	1.5	-	-	65	3.6±0.31	2.4±0.51
MS-19	2.0	-	-	20	2.5±0.25	2.1±.0.44
MS-20	-	0.5	-	-	-	-
MS-21	-	1.0	-	60	2.8±0.23	3.2±0.28
MS-22	-	1.5		44	1.5±0.32	3.4±0.51
MS-23	-	2.0	-	20	1.5±0.25	3.1±0.61
MS-24	-	-	0.5	-	-	-
MS-25	-	-	1.0	40	2.2±0.42	3.1±0.20

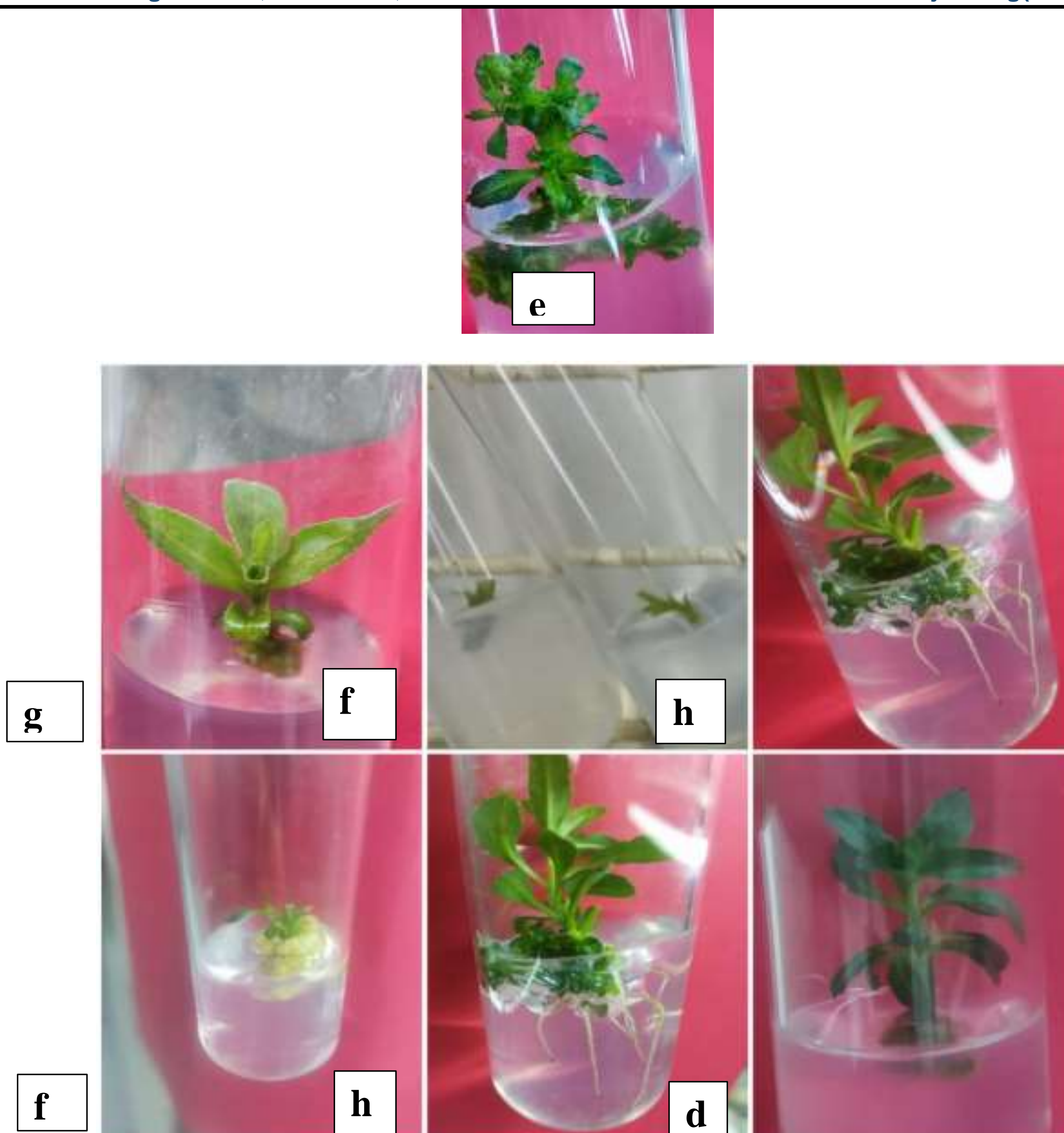
MS-26	-	-	1.5	40	2.8±0.23	2.6±0.15
MS-27	-	-	2.0	-	-	-

There were 6 shoots in each treatment and data (* ± SD) were recorded after 6 weeks of culture

Hardening

The well rooted plantlets transferred to the primary hardening mixture contain vermiculite and soil. Ninety percent of the survival rate was observed on primary hardening in the ratio of 1:1. It was observed that the gradual acclimatization is needed for the successful establishment in the secondary hardening stage. After 45 days of incubation, the primary hardened plants were transferred to cocopeat and sand mixture (1:1) for secondary hardening under the shade house (**Fig. 2h.**).

Figure 2. In vitro propagation of *Angelonia angustifolia Benth.*



a- Mother plant; b- Closed up view of explant and Shoot explant inoculated on medium; c- Shoot regeneration; d-Initiation e - Multiple shoot proliferation; f- sub cultured explant g – regeneration h- Healthy shoots with roots;

5. DISCUSSION

Plants are the producers in an ecosystem, and other organisms like animals, microbes and human beings are dependent on plants directly or indirectly. Plants have been the essential source for food, medicine, flavours, timbers and oils. Several plant species have been propagated conventionally by seed and also by vegetative means. To fulfill the need of increasing population, plant tissue culture technique is used as an alternative method of propagation (George and Sherrington, 1984).

Plant tissue culture is the science and art of growing plant cells, tissue, or organs isolated from the mother plant on artificial media. Tissue culture is a proven means of producing millions of identical plants under a controlled and aseptic condition, independent of seasonal constraints. Plant tissue culture plays an important role in the conservation of medicinal plants through rapid multiplication and rejuvenation of the endangered species (Tiwari Bhrigu Narayan *et al*., 2010).

The plant growth regulators play an important role in controlling the growth and development of plants. Auxin and cytokinin are the two important plant growth regulators widely used for morphogenetic manifestation under *in vitro* condition. The plant tissue culture technology has been very successfully used in large scale multiplication and has greatly contributed towards the upliftment of agriculture. Usage of tissue culture-generated plants has increased productivity per unit area, particularly in horticultural crops. Tissue culture has been one of the main technological tools and reasons that have contributed to the 'Second Green Revolution and Gene Revolution'.

In the present investigation nodal segments were used as explants in order to obtain shoot induction. The nodal segments were capable of directly producing multiple shoots on MS medium supplemented with various concentrations and combinations of cytokinin (BAP) and auxin (NAA).

The nodal segment was found to be the best explant for the initiation of shoots. The reason for the suitability of nodal segment is attributed due to the presence of protected axillary buds, which are not damaged during surface sterilization (Ajithkumar *et al*., 1998). A number of researchers suggested the nodal explants towards the successful propagation of other medicinal plants, such as *Rouwolfia serpentina* (Roy *et al.,* 1995), *Embilica officinalis* (Rahaman *et al.,* 1999), *Holarrhena antidyseterica* (Ahmed *et al.,* 2001) and *Enicostemma hyssopifolium* (Seetharam *et al.,* 2002).

In this investigation, shoot tip and axillary bud explants were inoculated on the medium containing MS salts supplemented with various concentrations of PGRs individually and in combinations towards the shoot induction. In the shoot induction phase different concentrations of BAP and in combination with various concentrations of NAA were used. All those concentrations supplemented to MS basal, medium were showed shoot induction but with varied shoot induction percentage, number of shoots and length of the shoots.

The possibility of direct regeneration from different explants including nodal and shoot tip explants of *A.angustifolia* has been evaluated on MS medium supplemented with various growth regulators. Among these BAP and NAA

combination proved to be efficient for inducing good number of shoots.

In this study, a reliable and reproducible protocol was developed with the help of growth regulators like BAP, IAA and NAA. Rapid multiplication of elite clones, production of healthy and disease-free plants and faster introduction of novel cultivars with desirable traits are of urgent need in crop improvement program. In the present study, a protocol for *in vitro* micropropagation of *A.angustifolia* from the nodal explants has been established to ensure the year round availability of high quality planting material.

The role played by cytokinins and auxins towards the *in vitro* morphogenesis is thoroughly established (Georg, 1993). Studies proved that nodal explants on BAP alone showed better response than with the other growth regulators viz NAA and IAA. Induction of multiple shoots was observed from axillary regions with BAP (2mg/l) + IAA (0.5 mg/l). After six weeks of inoculation, each explants produced an average of 7.1 ± 0.5 shoots with 80% response. Initiation of multiple shoots in most of the treatments was observed from 15th day onwards. Among all the combinations tested, the best response was noticed with 2 mg/l BAP. Explants inoculated with media supplemented with various other concentrations of BAP alone and also with IAA, NAA produced either clumps with highly-reduced shoots or callusing alone. Geetha *et al* (1998) found a similar response in *Cajanus cajan*, where multiple shoots were obtained on MS medium containing 2.0 mg L^{-1} Kinetin or BAP. Conversely, Bhat and Dhar, (2004) and Santarém and Astarita[14] found a similar response in *Myrica esculenta* buch- Ham. Ex. D. Don and *Hypericum perforatum* when sub cultured on low concentrations of KN. In the present study, lower concentration of BAP did not promote multiple shoots before and even after subsequent subcultures.

In this study, i.e. 1.0 mg/l of IAA concentration proved as a better one towards the induction of roots and it coincide with the earlier findings in palm (Tisserat, 1982), *Maesara mentacea* (Kanchanapooma and Boonvannob, 2000) and *Datura metal* (Muthukumar et al., 2001) and *Hybanthus ennaespermus* (Natarajan et al., 1999).

A more efficient micropropagation protocol was developed in this study. Though more number of multiple shoots was increased, the shoot length *A.angustifolia* was not improved by using same media

concentration. Even though, this protocol facilitated appreciable number of multiple shoots, it is not formed with sufficient shoot lengths. Quality roots (80% rooting) were induced with the help of half strength MS medium supplemented with 1mg/l IAA. This study establishes a simple, rapid, high frequency micropropagation method for *A.angustifolia* which can serve as basis for the *in vitro* propagation

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