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# Mass Propagation and Conservation of Three Important Medicinal Plants: Aloe Vera L., Acorus Calamus L. and Stevia Rebaudiana Bertoni

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University of Rajshahi

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# MASS PROPAGATION AND CONSERVATION OF THREE IMPORTANT MEDICINAL PLANTS: *ALOE VERA* L., *ACORUS CALAMUS* L. AND *STEVIA REBAUDIANA* BERTONI

A THESIS SUBMITTED TO
THE UNIVERSITY OF RAJSHAHI, BANGLADESH
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FOR THE DEGREE OF

*MASTER OF PHILOSOPHY*

IN

*BOTANY*

2007

BY

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DEDICATED

*TO*

*MY PARENTS*

# DECLARATION

I hereby declare that the entire work submitted as a thesis for the degree of Master of Philosophy to the Institute of Biological Sciences, University of Rajshahi, Bangladesh is the result of my own investigation.

**Supervisor**

29/06/07

**(Professor A K M Rafiul Islam)**

**Candidate**

Shahrear Ahmad

**(Shahrear Ahmad)**

# CERTIFICATE

This is to certify that the research findings, which are embodied in this thesis, are results of the experiments that have been carried out in Plant Breeding and Biotechnology Laboratory, Department of Botany, University of Rajshahi, Bangladesh under my supervision. The investigations are original and the results have not been submitted before in substance for any degree. It is further certified that the entire work presented as a thesis for the degree of Master of Philosophy is based on the results of author's own investigation.

**Supervisor** **Candidate**

29/06/07

(Professor A K M Rafiul Islam) (Shahrear Ahmad)

# ABSTRACT

The present investigation was undertaken to standardize *in vitro* culture techniques for mass propagation and conservation of two threatened important plant species, *Aloe vera* L. and *Acorus calamus* L. of Bangladesh and one recently introduced non-sugar natural herbal sweetener, *Stevia rebaudiana* Bertoni.

In case of *Aloe vera* through proper hormonal treatments it was possible to differentiate direct multiple shoots from shoot tip explants. The best multiple shoot production was achieved in MS+2.0 mg/l BA + 0.5 mg/l Kn + 0.2 mg/l NAA. The optimum pH level of the medium was found to be 6.0. As a carbon source, BDH was the best and local market Bangladesh sugar at 30-40 g/l was also found to give the satisfactory result. Agar at a level of 7 g/l irrespective of brands was highly effective to solidify media. For adventitious rooting of regenerated shoots $MMS_1$ + 0.2 mg/l NAA was proved to be the most favorable. The regenerated plants under *ex vitro* condition showed normal mode of development and 70% survivality was achieved when they were transferred to garden soil + compost (2:1).

For *in vitro* mass propagation of *Acorus calamus*, underground rhizome buds (rhizome tip) were used as explant sources. The explants were successfully cultured on MS medium with BA and Kn for primary shoot proliferation. Farther *in vitro* shoot multiplication was found to be the best in MS + 2.0 mg/l BA + 0.5 mg/l NAA. Optimum pH level was recorded 5.5, sucrose 40 g/l and agar 7 g/l. $MMS_1$ + 1.0 mg/l NAA was proved to be the best for rooting of micro shoots. The transplantation success was recorded 68% when the regenerated plants were transferred to soil-compost potting.

For *in vitro* mass propagation of *Stevia rebaudiana* nodal segments and of the pot grown mature plants were used for direct shoot proliferation while

leaf and inter-node explants from the *in vitro* grown shoots were used for adventitious shoot proliferation. MS medium having 1.0 mg/l BA + 0.1 mg/l NAA was proved to be the best for direct shoot proliferation from nodal explants. On the other hand, maximum adventitious shoot proliferation along with callus formation from leaf explants was achieved on MS medium containing 0.5 mg/l BA + 0.1 mg/l NAA. $MMS_1$ + 0.2 mg/l IBA was proved to be the best for adventitious root formation. Rooted shoots (plantlets) were gradually acclimatized and successfully established on the soil under natural condition with 65% survivality.

The methodologies developed in the present investigation are simple, highly effective and reproducible and can be utilized for *in vitro* mass propagation of three important medicinal plants- *Aloe vera* L., *Acorus calamus* L. and *Stevia rebaudiana* Bertoni.

The technique developed can be effectively used in bulking up planting materials of the newly introduced *Stevia rebaudiana* and the two endangered valuable medicinal plants, *Aloe vera* and *Acorus calamus* for their conservation and commercial exploitation.

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(Shahrear Ahmad)

# ABBREVIATIONS

The following abbreviations were used throughout the research paper:

| | |
|---|---|
| BA | 6-Benzyladenine |
| °C | Degree Celsius |
| *cf.* | *Confer* = please compare |
| cm | Centimetre (s) |
| $cm^2$ | Centimetre square |
| CW | Coconut water |
| e.g. | exampli gratia = for example |
| *et al.* | *et alia* = and other people |
| etc. | et celera = and the other |
| Fig. | Figure(s) |
| $GA_3$ | Gibberellic acid |
| g | Gram(s) |
| g/l | Gram per liter |
| i.e. | *id est* = that is |
| IAA | Indole-3-acelic acid |
| IBA | Indole-3-butylic acid |
| Kn | Kinetin (6-furfurylamino purine) |
| mg | Milligram(s) |
| mg/l | Milligram per liter |
| mg/ml | Milligram per millilitre |
| min. | Minute(s) |
| ml | Milliliter (s) |
| mm | Millimeter(s) |
| MS | Murashige and Skoog (1962) medium |
| $MMS_1$ | MS with $^1/_2$ strength of major salts but full strengtg of minor salts |
| $MMS_2$ | MS with $^1/_2$ Strength of both major and minor salts |
| $MSM_3$ | MS with full strength of major salts but $^1/_2$ strength of minor salts |
| NAA | α-Naphthalene acetic acid |
| No. | Number |
| 0.1N | 0.1 Normal solution |
| pH | Negative logarithm of hydrogen ion ($H^+$). |
| viz. | *videlicet* = Namely |
| v/v | Volume per volume |
| w/v | Weight per volume |
| % | Percentage |

# CONTENTS

**CHAPTER 2**

**MATERIALS AND METHODS**

# CHAPTER 1

# INTRODUCTION

## 1.1 Plant tissue culture: Concept and development

Plant tissue culture, an essential component of plant biotechnology, offers novel approaches to plant production, propagation and conservation. It is broadly defined as the growth, differentiation and maintenance of cells, tissues, organs or whole plants on artificial nutrient medium under *in vitro* condition. It is an important tool in both basic and applied sciences, as well as in commercial application (Bhojwani, 1990).

In recent times tissue culture technique has become an attractive field in biotechnological research throughout the world, especially in the areas of large scale clonal propagation, crop improvement through genetic manipulation, production of valuable chemicals, and conservation of plant genetic resources and generation of considerable variability among different types of plants.

Development of the science of tissue culture is historically linked to the discovery of the cell and subsequent propounding of the cell theory. Pioneering experiments on wound healing in plant, Henri-Louis Duhamel de Monceau's (1956) cited in Razdan (1993) demonstrated spontaneous callus formation on the decorticated region of elm plants is considered as the primary steps of plant tissue culture techniques. Cell doctrine, the next contribution to plant tissue culture, states that a cell is capable of autonomy and even has the potential of totipotency (Schlieden and Schwann, 1838-1839 *Cited in* De, 1992). Cellular totipotency is a concept that "all living cells containing a normal complement of chromosomes should be capable of regenerating the entire plant" (Haberlandt, 1902; *cf.* Krikorian and Berguam, 1969). Although Haberlandt was not successful in proving his idea about cellular totipotency but he was able to establish the foundation of plant tissue culture techniques through his series of experiments on different plants during 1900-1902.

The most popular application of plant tissue culture is micropropagation, which is an alternative to vegetative propagation of plants. It is now being used for large-scale multiplication of many plant species of diverse groups (Torres, 1988; Debergh and Zimmerman, 1990).

Tissue culture propagation might also prove useful for maintaining populations of rare and endangered species (Johnson and Emino, 1979 a, b,). *In vitro* clonal propagation via tissue culture is an effective method for large scale rapid multiplication of plants (Mott 1981, Rao and Lee 1982, Bajaj 1986). Through tissue culture over a million true to type plants can be grown from a small, even microscopic piece of plant tissue within a short period (Bhojwani and Razdan, 1983).

Plant tissue culture has a positive impact on the plant quarantine. It is utterly unlawful to transfer plants or plantlets to different countries for both the soil and the plant materials and supposed to carry microbes and in such cases plant quarantine will restrict their traffic. Whereas, *in vitro* plantlets since they are not the sources of contamination, are permissible and not restricted by plant quarantine laws.

Micropropagation the most popular of plant tissue culture method that allows production of large number of propagules in a relatively short period of time under controlled conditions, throughout the year in a relatively small space. The greatest success has been achieved in use of plant cell and tissue culture techniques with *in vitro* clonal propagation (Murashige, 1977; Shepard, 1982). Several pathogen free plants have been developed and plant tissue culture method now plays an important role in plant pathology (Rottier, 1978).

### 1.2 Micropropagation: Importance and application

Micropropagation technique has been the most significant commercial application of tissue culture technique with over 100 commercial facilities all over the world. *In vitro* micropropagation of plant has several advantages because of the following reasons (Murashige, 1978):

1. Propagation of clones all the year round independent of seasonal changes.
2. Large number of plants to be produced in a small space and short period of time under controlled conditions.
3. Genetically uniform plants can be produced and maintained in large numbers.
4. It is possible of *in vitro* storage for conservation by using (a) *in vitro* culture & propagation and (b) reducing the growth rate.
5. Superior gene combinations are propagated unaltered by cloning superior plants, which are lost through gene recombination in sexual method.
6. Improvement of gene pool of planting stock in sexual means is slow because of long breeding cycle whereas with cloning it is easy and much faster to get a true to mother type of a desirable clones.
7. Often the juvenile phase of development can be by passed if desired.
8. Some valuable hybrid and polyploid plants are infertile in general but they can be propagated by cloning through tissue culture.

Crop improvement is generally done through introduction, selection and hybridization. Normally these techniques of crop improvement are lengthy processes. Besides, use of conventional breeding methods for the improvement of crops is not very effective due to the heterozygosity and long life cycle in many plant species. Tissue culture technique provides viable alternative methods for micropropagation of healthy plants with uniform characteristics within a short period of time. Therefore, this technique can effectively be used in bulking up planting materials of the newly introduced, rare, endangered and threatened valuable plants.

**1.3 Demand of herbal medicine in worldwide**

Worldwide use of herbal pharmaceuticals is increasing at the rate of 15% against 3% for modern pharmaceuticals. In the United States of America

50% of herbal products used by the people are either in the form of health food (nutraceuticals), cosmaceuticals or as food supplements. In China more than 900 herbal drug manufacturing units are producing herbal products worth 18 billion Chinese Yan only for their domestic uses and the sale of these products are about 33% of total drug market globally i.e. 15 billion US$ per year (Report of task force on medicinal plants an action plan. Govt. of India, 1999).

There has been an increasing interest in herbal medicine not only in developing countries but also in developed countries. The global and national markets for herbal medicines and other herbal products are rapidly growing. There are about 45,000 plant species (including lower taxa) found in India of which about 7,500 are of medicinal value and nearly 120 plants are used in large scale by pharmaceutical industries. According to the secretariats of the Convention on Biological Diversity (CBD) there was US$ 60,000 million sales in the world herbal medicines and products market in 2000 which is growing at the rate of 7-15% annually and will become US$ 6 trillion by 2050 (World Bank Report, 1996).

Similarly in Japan the herbal market was of US$ 1,000 million in 1991, US$ 2000 million in 1994, US$ 2,200 million in 1996 and 2,400 million US$ in 2000. In United Kingdom herbal market was 92 million US$ in 1984, 134 million in 1998 , 159 million US$ in 2000 and US$ 200 million in 2004. About 147 herbal medicines are recommended by National Health Insurance Scheme in UK [WHO Guideline on Good Storage Practice (GSP) of Medicinal Plants, 2002].

There is now an increased demand for medicinal plants both for national medicinal use as well as for international trade. Asia is renowned for rich biodiversity and plant genetic resources. There is increasing exploitation of the wild resources, concomitant with increasing global trade. Medicinal plants have acquired increasing significance over the last decade or so, particularly after promulgation of the Convention on Biological Diversification. Medicinal

plants are not only important in respect of health care, but have a direct bearing on cross - sectoral issues such as biological diversity, environmental conservation, economic assistance, trade, intellectual property issues etc. The world market for plant derived chemicals, cosmetics, pharmaceuticals, fragrance, flavors and colour ingredients, alone exceeds several billion dollars per year. It is estimated that the global trade in herbal products is US$ 60 billion (WHO, 2002).

The botanical market, inclusive of herbs and medicinal plants in the USA, is estimated, at retail at approximately US$ 1.6 billion p.a. China with exports of over 120,000 tones p.a. and India with some 32,000 tones p.a. dominate the international markets. It is estimated that Europe, annually imports about 400,000 tones of medicinal plants with an average market value of US$ 1 billion from Africa and Asia.

In late 1997, the world bank, within the framework of the Global Environmental Facility, provided a US$ 4.5 million grant for the "Srilanka Conservation of Medicinal Plants Project" which focuses on the conservation of medicinal plant populations, their habitats and their sustainable use in Medicinal Plant Conservation Areas (MPCAs). Inventories with emphasis on the management, research and conservation of rare and endangered species of medicinal plants are the main program at MPCAs at Ritigala, Naula, Rajawaka, Kanneliya and Bibile.

Thus the demand of medicinal plants has placed a great strain on the natural populations of plants. Collectors of medicinal plants are now resorting to unsustainable exploitation causing serious threat to the survival of some species. Dwindling supplies are exacerbated by the rapid depletion of natural habits. Forest degradation has occurred particularly rapidly in the biodiversity-rich, S-E Asian countries. Deforestation and forest degradation have resulted in the significant loss of biodiversity in general and medicinal plant resources in particular. This loss has led to the erosion of valuable traditional knowledge

and practices of the minorities in using these medicinal plant resources for disease treatment. The Centre for Science and Technology of the Non-Aligned and other Developing Countries in India organized an international workshop on "Tissue culture of economic plants" in April, 1994, as a means of using modern biotechnological techniques to nurture and conserve medicinal plants.

### 1.4 Herbal drugs in Bangladesh

Bangladesh has a great treasure of medicinal plants spread over an area of about 144,000 sq. km. and endowed by nature with a very favourable climate high humidity, annual rain falls about 120-508 cm, average temperature flicks with 9-36°C (winter to summer). Bangladesh possesses what is perhaps one of the richest floras of all other areas of a similar size on the surface of the globe. A great variety of plants grow in its forests, jungles, wastelands and in the roadsides. It is not surprising therefore that plant containing active and medicinal principles grown abundantly within its bounds. More than 500 plants have been reported to have medicinal properties of some description or other and have been enumerated in the literature of indigenous drugs. A good number of the natural drugs included in different pharmacopoeial and unofficial medicinal plants grow here wild, many others can be easily grown.

Almost all these indigenous medicinal plants are extensively used in the preparation of Unani, Ayurvedic and Homeopathic medicines in Bangladesh. In this regard it is noticeable that the Hamdard Centres have developed into the largest facilities in the world for the study, treatment and research of Unani therapeutics. Hakim Mohammed Sayed established Hamdard in Pakistan and Bangladesh. Further Hakim Md. Yousuf Haroon Bhuiyan, Managing Director of Hamdard Bangladesh, made a breakthrough in the history of Unani medical science in Bangladesh. With his colleagues he has succeded to get the official recognition of Eastern Medicine in Bangladesh (Islam, 2003).

His utmost effort made it possible to reintroduced Unani Medicine as the bridge between traditional medicine and modern medicine. At present the practitioners of modern medicine and traditional medicine prescribes Hamdard medicine in their routine practice for he common and complicated ailments. Today Hamdard Bangladesh has introduced time honored presentation of Unani Medicine like capsule, tablet, syrup. A study has shown that about 40% of the best selling drugs were either biological, natural products or entities derived from them (Laird and Kate, 2002)

Under the proper guidance of Justice Abdur Rouf (Chairman Hamdard Board of Trustees), National Professor, Dr. Nurul Islam (Vice-Chairman, Hamdard Board of Trustees) and other honourable members of the Board of Trustees, Hakim Md. Yousuf Haroon Bhuiyan has started activities to set up a university "City of science, Culture and Education" at Biggam Nagar in Sonargaon. The prime subject of the University will be Eastern Medicine, Modern Medicine, Information Technology, Biotechnology, Genetic Engineering, Applied Botany and Phytochemistry.

Hakim Md. Yousuf Haroon Bhuiyan succeded to set up a modern factory in Sonargaon, to produce and marketing modern herbal medicine. The annual production capacity of that factory is about 500 crore taka. It has introduced time honoured presentation of Unani Medicine like-capsule, tablet, syrup etc. He established Research and Development Department and started extensive research work in association with his colleagues and scientists.

Recently, Hamdard has acquired 300 acre land in Chittagong for cultivation, conservation and propagation of rare and valuable medicinal plants, which will save million dollars of foreign currency used in importing raw materials. On the other hand, substantial amount of foreign currency may be earned by exporting. Because Bangladesh has several unique features in the medicinal and aromatic plants (MAP) sectors, the first and most important being wider use of native plants in the Bangladesh systems of indigenous

medicine which still cater to a large section of the people. Out of the estimated 500 species of phanerogams growing in this country more than a thousand are regarded as having medicinal properties (Ghani, 1998). So, government of Bangladesh and some big non-government organizations BRAC, Proshika Square Pharma, Beximco Pharma in the country may come heading towards this direction like Hamdard.

## 1.5 The experimental plant-*Aloe vera* L.

### 1.5.1 Description of experimental plant *Aloe vera*

*Aloe vera* L. is a xerophytic perennial herb with a rosette of long thick lanceolate leaves with spiny margins, occasionally grown in gardens as an ornamental plant (Ghani, 1988). Most botanists agree and historical evidence suggests that the *Aloe vera* plant originated in the warm, dry climates of Africa. In the United States, it is grown commercially in the Rio Grande Valley of Texas, in California and Florida, and in specially-designed Green Houses in Oklahoma state (source: http://www.dansaep.com/AboutAloe.html).

### 1.5.2 Systematic position

Systematic position of *Aloe vera* according to Cornquist (1968) is mentioned below:

Division : Angiosperm
Class : Monocotyledonae
Order : Liliales
Family : Liliaceae
Genus : *Aloe*
Species : *Aloe vera* L.
Common name- Ghritakumari, Musabbar (Beng.), Aloe (Eng.)

### 1.5.3 Phytochemicals in *Aloe vera*

Principal constituents of leaves of *Aloe vera* are anthraquinone glycosides, called aloins which include barbaloin, iso-barbaloin, β-barbaloin, emodin, chrysophanol, chrysophanic acid and aloe-emodin, uronic acid and enzymes. Resins, sterols, triterpenes, cuomarins, saponins, carbohydrates, amino acids and vitamins are also present in this valuable plant.

The *Aloe vera* plant produces at least six antiseptic agents: lupeol, salicylic acid, urea nitrogen, cinnamic acid, phenol and sulfur. All these substances are recognized as antiseptics, because together they exhibit antimicrobial activity. This explains why *Aloe* has the ability to eliminate many internal and external infections. Lupeol, salicylic acid, and magnesium are also highly effective analgesics, and this explains why *Aloe* is an effective pain killer.

### 1.5.4 Uses of *Aloe vera*

**Beauty treatment:** *Aloe vera* has a long history as a skin lotion-Cleopatra is said to have attributed her beauty to it.

**Western remedy:** In the West, *Aloe vera* first became popular in the 1950s when its ability to heal burns in particular radiation burns, was discovered.

**First aid:** *Aloe vera* is an excellent first aid remedy to keep in the home for burns, scrapes, scalds and sunburn. A leaf broken off releases soothing gel, which may be applied to the affected part.

**Skin conditions:** The gel is useful for almost any skin condition that needs soothing and astringing, and will help varicose veins to some degree.

**Ulcers:** The protective and healing effect of *Aloe vera* also works internally, and the gel can be used for peptic ulcers and irritable bowel syndrome.

**Laxative:** The bitter yellow liquid in the leaves (bitter aloes) contains anthraquinones which are strongly laxative. They cause the colon to contract, generally producing a bowel movement 8-12 hours after consumption. At low doses, the bitter properties of the herb stimulate the digestion. At higher doses, bitter aloes are laxative and purgative.

**Other medical uses:** *Aloe* is a cathartic and used in correcting habitual constipation. It is also used as emmenagogue and anthelminmtic and in the treatment of jaundice, loss of appetite, gas formation in the stomach, leucorrhoea, menstrual suppression, burning during sexual ejaculation, piles, rectal fissures, inflammations, ulcers, burns, scalds, abscess, acne, balanitis, cervical cancer, herpes, lung cancer, mouth ulcers and wrinkles (source: http://www.herbs2000.com/herbs/herbs_aloe_vera.htm).

In Greek pharmacology, the plant was first mentioned by Celsius (B.C. 25-50 A.D.) but his comments were limited to its power as a purgative. The first western benchmark in man's understanding of Aloe in the Greek herbal of Dioscorides (41 A.D.-68 A.D.). The master of Roman Pharmacology developed his knowledge and skill as he traveled with that great empire's armies. Dioscorides gave the first detailed description of the plant we call *Aloe vera,* and attributed to its jucie " the power of binding, of inducing sleep." He noted as well that it "loosens the belly, cleansing the stomach." He further added that this "bitter" *Aloe* (the sap) was a treatment for boils; that it eased hemorrhoids; that it aided in healing bruises, that it was good for the tonsils, the gums, and all general mouth irritations; and that it worked as a medicine for the eyes. Dioscorides further observed that the whole leaf, when pulverized, could stop the bleeding of many wounds (Source: http://www.dansacp.com/AboutAloe.html).

As we noted it that everything old is new again. In each culture, *Aloe vera* has drawn the attention of the most sophisticated of minds. At present, *Aloe vera* has little official standing in the medical community. In spite of the lack of official promotion, it is among the most widely used substances in the

U.S.A. for the treatment of burns and bruises. Additionally, it is used in a plethora of cosmetics and consumed as a healthful drink. While it has not yet won the full endorsement of the medical community, serious examination continues. For the moment, it may be taken as an indication of the serious nature of such a study that the FDA has approved development aimed at the eventual use of *Aloe vera* in the treatment of Cancer and AIDS more and more, attention is turning to Aloe's unexamined possibilities as a powerful healing agent.

## 1.6 The experimental plant-*Acorus calamus* L.

### 1.6.1 Description of experimental plant- *Acorus calamus*

*Acorus* is a genus of herbs and is found in northern temperate and subtropical regions. The plant is distributed throughout Indian subcontinent in marshy, wild habitats or cultivated, ascending the Himalayas upto 6,000 ft. in Sikkim, i.e. in north temperate and warm regions (Kirtikar and Basu, 1989).

The sweetflag or calamus (*A. calamus*) of the family Araceae have been used as experimental materials. In Bangladesh it is known as Boch.

Sweetflag is a perennial herb with long creeping and aromatic rhizomes or under ground stems sprouting leaves. The flowering shoots of the plant are sprouted by a large leaf like structure called spathe. It has pale-green, small flowers, in 5 to 10 cm long cylindric spikes and yellowish fruits (Bakhru, 1993). Rhizome of calamus horizontal, jointed, somewhat vertically compressed, spongy within, 1.25-2.5 cm thick, pale to dark brown or occasionally orange-brown in colour, leaves sprouted from the rhizome, grass-like or sword shaped, long and slender; flowers small, yellow-green, in a spathes; barriers green, angular, 1-3 seeded; seeds oblong (Chadha, 1985)

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### 1.6.2 Systematic position

Systematic position of sweetflag (Boch) according to Cronquist (1968) is mentioned below:

| | |
|---|---|
| **Division** | : Magnoliophyta |
| **Class** | : Liliatae |
| **Sub class** | : Arecidae |
| **Order** | : Arales |
| **Family** | : Araceae |
| **Genus** | : *Acorus* |
| **Species** | : *A. calamus* L. |

### 1.6.3 Propagation

In conventional method, sweetflag is propagated vegetatively. It can be propagated by rhizome sections and also through seedling transplantation.

### 1.6.4 Cultivation and harvesting

The plant is grown in clay loams and light alluvial soils of riverbanks. The field is irrigated and ploughed with green manure before planting. The growing ends or tops of previous year's crop are planted 30 cm apart, leaving the leafy portions well above the ground. The crop is ready for harvest in about a year. The plants are dug out, rhizomes removed, and the tops kept for the next planting. The rhizomes are cut into pieces of 5-8 cm and all fibrous roots are removed. The pieces are washed thoroughly and dried in the sun. the dried material is put into rough gunny bags and rubbed to remove the leafy scales. The yield of rhizome is 3,750 kg/ha and with proper cultivation, almost double the yield is possible (Chopra *et al.*, 1958 cited in Chadha, 1985). In Bangladesh it is grown in limited scale, generally in the marshy places or near the homestead drainage lines, wild or under cultivation in Chittagong and Chittagong Hill Tracts.

### 1.6.5 Chemical composition

Several parts of *A. calamus*, rhizomes roots and leaves yield volatile oil known as calamus oil. The yield of oil from different parts of the plant is as follows: fresh rhizome, up to 1.8%; dried rhizomes 1.5-3.5%; leaves 0.2%; and fresh aerial parts 0.12%. The highest yield of oil is obtained from dried, unpeeled rhizomes (Trivedi and Mann, 1988; Chopra *et al.*, 1958, *cited in* Chadha, 1985). The important constituents of the Indian calamus oil are asarone (up to 82%) and its β-isomer. Other constituents are calamenol 5.0%, calamene 0.4%, calamenone 1.0%, methyl eugenol 1.0%, engenol 0.3%, α-pinene and camphene 0.2%. Choline (0.26%) and acric acid have been isolated from the roots. A bitter aromatic principle, acorin and a flavone diglycoside, have been separated from the plant. Tannins, mucilage and calcium oxalate are also reported (Chadha, 1985; Ghani, 1998).

### 1.6.6 Use and importance

The root of *A. calamus* has been employed in medicine since the time of Hippocrates. However, it is successfully used by the modern physicians in the treatments of intermittent fevers, stomachic infusions, and coughs or sore throats. The root has been found extremely useful in the dysentery of children and also in bronchitis affections. In Srilanka, the rootstock is used in bowel complaints. The root is supposed by the Chinese to affect the heart and lungs and to be beneficial for cancer. In general, it is taken as a restorative for the body and spirits. In Constantinople, the root is eaten as a preventive against pestilence. The Indians of the Hudson's Bay Territory use the root in coughs. The Europeans of South Africa use the rootstock as a carminative and as a diarrhea remedy (Kirtikar and Basu, 1989). The rhizome is pungent, bitter, heating; emetic, laxative, diuretic, carminative, anthelmintic; improves appetite, voice, throat; good for disease of the mouth; useful in abdominal pains, inflammations, fevers, epilepsy, bronchitis, delirium, hysteria, dysentery, tumours, thirst, loss of memory, rat-bite, worms in the ear (Chadha, 1985).

The rhizome has a very bitter sharp taste, laxative, expectorant, carminative, alexiteric tonic to the brain, emmenagogue, useful in general weakness, stomatitis, toothache, inflammations, pains in the liver and the chest, kidney troubles, leucoderma (Kirtikar and Basu, 1989).

### 1.7 The experimental plant-*Stevia rebaudiana* Bertoni

*Stevia*, a natural sweetener plant which contain stevioside. Stevioside making up to 6% to 18% of constituents of *Stevia* leaves, is also the most prevalent glycosider present in the leaves. Stevioside is considered 300 times sweeter than sucrose at 0.4% sucrose and 100 times sweeter at 10% sucrose concentration. It has also been shown not to be mutagenic, genotoxic nor did it produce effects on fertility. It is native to the valley of the Rio Monday in highland of Paragauy (source: http://www.rain-tree.com/*Stevia*.htm). Its hierarchical position, morphology and distribution are given below:

#### 1.7.1 Hierarchical position (According to Cronquist, 1968)

| | | |
|---|---|---|
| Plant kingdom | : | Plantae |
| Division | : | Magnoliophyta |
| Subdivision | : | Magnoliphytina |
| Class | : | Rosopsida |
| Sub class | : | Asteridae |
| Super order | : | Asteranae |
| Order | : | Asterales |
| Family | : | Asteraceae |
| Tribe | : | Stevieae |
| Genus | : | *Stevia* |
| Species | : | *S. rebaudiana* Bertoni |

### 1.7.2 Morphological characters

This plant is a much branched herb in the first year of growth but it becomes shrubby while perennial. It grows up to 1 meter tall and has 2-3 cm long leaves. Leaves are simple, opposite, obate to globuse, margin serrate, yellow-green, petiolate, stem round and hairy. The inflorescence is a small whitish capitulum.

### 1.7.3 Geographical distribution

*Stevia* is a perennial shrub belonging to the aster family, which is indigenous to the northern regions of South America. It grows up to a meter tall and has 2-3 cm long leaves. It is still found growing wild in the highlands of the Amambay and Iguacu districts (a border area between Brazil and Paraguay), however it is now commercially grown in parts of Brazil, Paraguay, Uruguay, Central America, Isreal, Thailand and China (source: http://www.rian-tree.com/*Stevia*.htm).

### 1.7.4 Propagation and cultivation

It is a short day plant, growing up to 0.6 metre in the wild and flowering from January to March in the Southern Hemisphere. Flowering under short day conditions should occur 54-104 days following transplanting, depending on the day length sensitivity of the cultivars.

*Stevia* grows naturally on infertile, sandy acid soils with shallow water tables. This is normally grown in areas like the edge of mashes and grassland communities. The natural climate is semi-humid subtropical with temperature extremes from 21° to 43°C, averaging 24°C. *Stevia* grows in areas with up to 1375 mm of rain fall in a year.

Reproduction in the wild is mainly by seed, but seed viability is very poor and highly variable. *Stevia* can be produced by cutting but once good varieties have been created, a seed/transplant production system is more economic.

Under cultivation, *Stevia* plants can achieve a height of 1 meter. In agronomic conditions, the plant is normally treated as an annual. Seeds are germinated in the glasshouse in spring and the plants are transplanted in to the field (usually at 6-7 weeks old) (source: http: //www.newcrops.uq.edu.au/newslett/ncn11161.htm).

### 1.7.5 Phytochemicals

*Stevia* leaves contain diterpene glycosides, the leaves also produce active substances which are biologically important e.g. flavonoids, coumarins, cinnamic acids and essential oil. Also this plant contains many chemical components such as stevoil, stevioside, rebaudiosides, aluminum, ascorbic acid, ash, austroinulin, beta-carotene, calcium, chromium, cobalt, dulcosides, iron, magnesium, manganese, niacin, phosphorus, potassium, protein, riboflavin, selenium, silicon, sodium, thiamin, tin, zine (source: http://www.rain-tree.com/*Stevia*.htm).

### 1.7.6 Properties and uses

*Stevia rebaudiana* is a herbaceous perennial plant. It is extremely sweet, non caloric and normally used as a natural harbal sweetener. The leaves of *Stevia rebaudiana* contain stevioside a natural sweetner, 300 times sweeter than sugar. The plant was first studied scientifically in 1899 by Parayauyan botanist Moises S. Bertoni, who recognized the plants incredible sweetening power. He suggested that "stevioside might substitute saccharine as a sweetening agent being completely non-toxic". *Stevia* is also considered to be a great agent in weight loss programmes because it is very low in calories and its sweetness is so concentrated that chewing a few leaves of *Stevia* will satisfy anyone's sweet tooth, and the shredded leaves are an excellent substitute for sugar in cooking, as well as in tea and coffee preparations. Being a natural herbal sweetener it is useful in treating the symptoms of diabetes, high blood pressure and infection (source: http://www.rain-tree.com/*Stevia*.htmp-6).

*Stevia* is also used by the Japanese to sweeten a variety of food products, including ice cream, bread, candies, pickles, seafood, chewing gum, vegetables and soft drinks (http://www.*Stevia*.net/history.htm). People in Brazil and Paraguay have used the leaves of *Stevia* as a sweetener. The Guarani Indian of Paraguay call it as "Kaa Jhee' and have used it to sweeten their Yearba Mate tea for centuries. They have also used it to sweeten other medicinal teas and foods as well as use it medicinally as a cardiotonic, diabetes, diuretic, high blood pressure, hypoglycemic, hypotensiva sweetener, tonic, vasodilator, heart burn, and to help lower uric acid levels (source: http://www.rain-tree.com/*Stevia*.htm). In China *Stevia* and stevioside are used in herbal medicines and toothpaste. In China, Korea and Japan *Stevia* extract containing stevioside is used as a table top sweetener because it has zero calories, zero carbohydrates, zero fat and is reportedly a natural sweetener as 300 times sweeter than sugar (source: http://www.*Stevia*-stevioside.com/*Stevia*_rebuadiana_bertoni.htm).

Some "folk" uses (Primary in Latin American and the Orient) include the following: stimulate alertness and counter fatigue; facilitate digestion and gastrointestinal functions; regulate blood glucose levels; nourish the liver, pancreas and spleen; and external application for blemishes. *Stevia* extract has been shown in studies to inhibit the growth and reproduction of some bacteria that are responsible for tooth decay (source: http://www.*Stevia*-stevioside.com/*Stevia*_rebaudiana_bertoni.htm).

Nearly 20 years, Japanes and Brazilian consumers by the millions have used *Stevia* extracts as a safe, natural, non-caloric sweetener where it is approved as a food additive. Japan is the largest consumer of *Stevia* leaves and extracts in the world where it is used to sweeten everything for soyasauce, pickles, and confections to soft drinks. Even multi-national giants like Coca-Cola and Beatrice foods use *Stevia* extracts to sweeten foods (as a replacement for nutra sweet and saccharin) for sale in Japan, Brazil and other countries where it is approved as a food additive (source: http://www.rain-tree.com/*Stevia*.htm).

*Stevia* can also help the body sustain a feeling of vitality and well being. Users have also reported that drinking *Stevia* tea or *Stevia* enhanced teas helped to reduce their desire for tobacco and alcoholic beverages. Recently there have been studies linking *Stevia* extract with normalization of blood sugar, and insulin in diabetes (source: http://www.*Stevia*-stevioside.com/*Stevia*_rebaudiana_bertoni.htm).

**1.8 Tissue culture of medicinal plants**

Medicine and aroma constitute an important group of chemicals originated both from the wild and cultivated plants. Accordingly, WHO has formulated a definition of medicinal plants in following ways: "A medicinal plant is any plant which is one or more of its organs, contains substances that can be used for therapeutic purpose or which is a precursor for synthesis of useful drugs" (Sofowora, 1982 cited in Ghani, 1998).

In the recent period plant tissue culture or the *in vitro* culture of plant cells, tissues and organs is considered as an important tool in both basic and applied researches under the umbrella of plant biotechnology. Plant tissue culture has a wide range of commercial applications. *In vitro* micropropagation of medicinal plants has a number of advantages over the sexual and conventional methods because of the following reasons:

a) Selected novel genotypes of medicinal interest, which are lost through gene recombination in sexual method, can be propagated unaltered by cloning *in vitro*.

b) It is easy and much faster to get a true-to-mother type of a desirable clones as improvement of gene pool in suxual means is slow.

c) Genetic uniformity of a clone is maintained.

d) In some species, clonal propagules initially grow much faster than seedlings.

e) Some valuable hybrid and polyploid plants are infertile in general, but they can only be propagated by cloning through tissue culture.

f) In relatively short time and space a large number of plants can be produced starting from a single individual or even from an explant of a selected clone and can be continued all the year round.

g) Stable variability can also be created in relatively short time in a target genotype using callus and cell cultures through somaclonal variation.

The important species, specially medicinal plants, for which *in vitro* method of propagation has been adopted are mentioned below with success achieved in each case.

Multiple shoot formation was induced on callus from stem segments of *Plumbago rosea* on MS medium containing auxin and cytokinin. Addition of 2,4-D (2.5 mg/l) and kinetin (1.5 mg/l) to the medium was found to be best for callus production while BAP (2.0 mg/l) plus NAA (1.0 mg/l) was proved to be suitable for induction of shoot formation from that callus. Numerous shoots with roots could be produced by transferring the shoots to a medium containing IBA (1.5mg/l). Regenerated plantlets were transferred to pots and 60% of them survived (Kumar and Bhavanandan, 1988).

Petiole explants of *Valeriana wallichii* were used for callus induction. Optimum callus formation was found on MS medium supplemented with NAA (0.3 mg/l) and kinetin (0.25 mg/l). Shoot regeneration was achieved by transferring the callus to medium containing kientin (1.0 mg/l) and NAA (0.25 mg/l). Complete plantlets were obtained by producing roots on the shoots on the same medium. Callus regenerated plants were successfully transferred to the field following hardening procedures (Ahuja *et al.*, 1989; Mathur and Ahuja, 1991).

Micropropagation of *Coleus forskohlii* was achieved using nodal segements cultured on MS medium supplemented with kinetin (2.0 mg/l) and IAA (1.0mg/l). Shoots were multiplied at a rate of 12-fold per every 6 weeks. Rooting occurred when shoots were transferred to MS medium containing IAA

(1.0 mg/l). Regenerated plants were successfully transferred to the field (Sharma *et al.*, 1991).

Shoot tip explants of *Rheum emodi* were cultured on MS medium containing BAP (2.0 mg/l) and IBA (1.0 m/l) produced multiple axillary shoots while adventitious shoot buds developed form leaf explants on MS medium with BAP (2.0 mg/l) and IAA or IBA (0.25-1.0 mg/l). Roots were induced when these shoots were placed on MS medium with IBA (1.0 mg/l). Both regeneration procedures gave rise to healthy plantlets that were established on soil under green house conditions at 80% success after a hardening phase of 2 weeks (Lal and Ahja, 1989).

Leaf and stem segments of *Gomphrena officinalis* from aseptically grown seedlings were used to initiate cultures. Callus production was found on agar gelled MS medium supplemented with BA alone (1.0, 5.0 or 10.0 mg/l) after 10-15 days of culture. The best shoot regeneration was found with the combination of BA (5.0 or 10.0 mg/l) with NAA (0.1 mg/l). Adventitious shoot formation occurred on leaf and internode explants after 50-60 days of culture. Nodal segments developed actively growing lateral buds after 30 days of culture. The best root formation was observed on MS medium containing IBA (10.0 mg/l). The plantlets were transferred to potting soil and were successfully established under natural environment (Mercier *et al.*, 1992).

*Rauvolfia serpentina* Benth is one of the most important medicinal plants. Stem explants of this plant were cultured on MS medium supplemented with NAA (1.0 mg/l), Kn (0.5 mg/l) and 1% CM for inducing callus. Shoot buds were regenerated from the callus after 20 weeks on White's medium containing CM (100.0 ml/l), biotin (10 ml/l), SDDC (250 mg/l) and NAA (0.8 mg/l). The shoots were rooted on White's medium containing 3 mg/l each of IBA and NAA. Similarly callus obtained from roots were regenerated on White's medium containing BA (2.0 mg/l) and NAA (0.8 mg/l). The plantlets were transferred to the field after acclimatization (Ilahi, 1993).

There are many other medicinal plant species for which tissue culture techniques have been used for regenerating plantlets *in vitro*. The species and authors' names are mentioned bellow.

*Adhatoda vasica* (Amin *et al.*, 1997), *Aegle malmelos* (Arya *et al.*, 1989; *Hossain et al.*, 1995), *Agave sisalena* (Savangikar *et a.*, 1989), *Allium sativum* (Singh *et al.*, 1989), *Asclepias curassavica* (Pramanik and Datta, 1986), *Atropa belladonna* (Ahuja *et al.*, 1989), *Catharanthus roseus* (Fulzele *et al.*, 1989), *Capsicum annuum* (Venkataraman *et al.*, 1989) *Crataeva nurvala* (Sharam and Padhya, 1989), *Cymbopogon winterianus, C. flexuosus, C. mortinii* (Mathur *et al.*, 1989), *Dioseorea floribunda* and *D. deltoids* (Ahuja *et al.*, 1989; Sharma and Chaturvedi, 1989), *Echinacea angustifolia* (Goeckel *et al.*, 1992), *Emblica officinalis* (Kant *et al.*, 1989), *Hyoscyamus muticus, H. albus* and *H. niger* (Ahuja *et al.*, 1989), *Mentha arvensis* (Kukreja *et al.*, 1991), *Nicotiana tabacum* and *N. rustica* (Reddy, 1989), *Ochrosia elliptica* (Ramawat *et al.*, (1989), *Panax ginseng* (Chang and Hsing, 1980; Chio *et al.*, 1984; Lee *et al.*, 1991), *Peganum harmala* (Nag *et al.*, 1989), *Phaseolus vulgaris* (Reddy, 1989), *Picrorhiza kurroa* (Ahuja *et al.*, 1989), *Podophyllum hexandrum* (Arumugam, 1989), *Pogostemon patchouli* and *P. cablin* (*Ahuja et al.*, 1989), *Solanum nigrum, S. xanthocarpum, S. jasminoides and S. verbascifolium* (Singh *et al.*, 1989; Mehta, 1989; Subramani *et al.*, 1989; Jain *et al.*, 1989), *Tecomilla undulata* (Arya *et al.*, 1989), *Terminalia belerica* (Roy *et al.*, 1987), *Tribulus alatus* (Nag *et al.*, 1989), *Trigonella foenumgraccum* (Jain *et al.*, 1989), *Ziziphus mauritiana* (Arya *et al.*, 1989) and *Zygophyllum simplex* (Nag *et al.*, 1989). *Elettaria cardamomum* (Nadgauda *et al.*, 1983), *Zingiber officinale* (Hosoki and Sagawa, 1977), *Curcuma domestica* (Nadgauda *et al.*, 1978) and *Allium sativum* (Singh *et al.*, 1989) etc. As far as we know there is no reports in Bangladesh on *in vitro* mass propagation of *Aloe vera* L., *Acorus calamus* L. and *Stevia rebaudiana* Bertoni, three important medicinal plants and the last one is a potential new crop for Bangladesh.

## 1.9 Advances in tissue culture of the experimental plants

One of the main applications of microprogation is mass propagation of superior plants. In India microprogation has been started in 1982 with the standardization of mass propagation techniques (Villaman *et al.*, 1996).

### 1.9.1 *Aloe vera* L.

There have been some reports from other countries on tissue culture of *Aloe vera.* Wang *et al.* (2002) in China observed that MS medium supplemented with 1 mg/l BA was optimum for adventitious bud differentiation. They used adventitious buds as explant sources.

A method for *in vitro* propagation of *A. vera* was developed by Zhou *et al.* (1999). The best medium for the induction of buds was MS medium (Murashige and Skoog, 1962) supplemented with BA (benzyl adenine) at 3 mg/l, and the best rooting was observed in presence of NAA at 0.3 mg/l.

Using the underground rhizomatous stem Roy and Sarker (1991) propagated *Aloe vera* at large scale in India. They used 1g/l polyvinyl-pyrrolidone in the culture medium to reduce secretion of phenol substances by the explants. They used MS basal medium for induction of callus with 1 mg/l 2, 4-D and 0.2 mg/l kinetin. Shoot initiation was achieved on media containing 1 mg/l kinetin with 0.02 mg/l 2, 4-D. Chinese scientists Gui *et al.* (1990) cultured *Aloe vera* stem segments on MS medium. They used hormone of 2 PPM zeatin plus 0.5 PPM NAA and observed that plantlets were derived from cell masses formed on the surface of the callus.

SriLankan scientists Hirimburegama and Gamage (1995) used meristem tips as an explant source of *A. vera.* They used MS medium with 2.25 mg/l BA plus 0.18 mg/l IAA for mass propagation. The rooting was achieved by incubation on MS medium with 0.18 mg/l IAA + 0.22 mg/l BA for 3 weeks followed by MS medium with 0.02 mg/l IBA.

### 1.9.2 *Acorus calamus* L.

A method for micropropagation of *Acrous calamus* was described by Harikrishnan *et al.* (1997). MS solid medium was proved to be more effective than MS liquid medium. They cultured young rhizomatous buds in different concentrations of BAP and NAA containing medium with 0.5 mg/l BAP and 0.05 mg/l NAA was found most effective for inducing shoots.

Harikrishnan *et al.* (1999) used young rhizome buds of *A. calamus* as an explant source. Shoot initiation was achieved on MS (Murshige and Skoog, 1962) media containing 0.5 mg/l BA plus 0.5 mg/l NAA. They further observed that addition of activated charcoal (0.2%) into the medium inhibited phenotic exudation and the browning of the medium. The rooting from microshoots was achieved by incubation on MS medium with 1.5 mg/l NAA and 0.2% activated charcoal.

### 1.9.3 *Stevia rebaudiana* Bertoni.

Bespalhok *et al.* (1992) used 2 cm long nodal segments excised from the adult *Stevia rebaudiana* plants and cultured for shoot proliferation on MS medium containing 6 levels of $NH_4NO_3$ De Fossard vitamins, 5.0 µM BA, 3% sucrose and 1% agar.

Bespalhok *et al.* (1993) induced somatic embryos from leaf explants of *S. rebaudiana* on MS medium supplemented with 2, 4-D plus BA and high sucrose concentration (120 g/l).

Bespalhok and Hattori (1997) reported that somatic embryos were obtained from floret explants on MS medium supplemented 2,4-D (9.05 or 18.10 µM) and kinetin (0-9.29 µM). They also observed embryonic callus formation on 9.05 µM 2,4-D supplemented medium and callus formation started at the base of the corolla and ovaries.

Bondarev *et al.* (1998) observed that callus formation of leaf blade was 2 to 3 times higher than that of stem segments and NAA was found more effective for cell growth than 2,4-D, while BA exhibited a stronger effect than kinetin.

## 1.10 Objectives

*Aloe vera* is used as emetic and anthelmintic and in the treatment of jaundice, loss of appetite, gas formation in the stomach, leucorrhoea, menstrual suppression, piles, rectal fissures, inflammation-ulcers, burns and scalds (Ghani 1998). As the plant in extensively used on herbal medicine and cosmetic industry, its demand is dramatically increasing. The plant in generally propagated by means of suckers arising from the base of mother plant. The natural vegetative propagation of *Aloe* is very slow. Once upon a time in own nature it was found in groves and jungles but today it is near about a threatened plant species in our nature (Kormaokar 2003). So an alternative method might be the use of *in vitro* propagation for rapid multiplication and conservation of *Aloe vera.*

On the other hand, the medicinal plants those with rhizomatous, aromatic stems are more vulnerable due to uprooting of the plants for ethno-medicinal purposes. Khan *et al.* (2001) described *Acorus calamus* is a threatened plant species. The application of *in vitro* techniques for propagation and conservation of the plants either with elite genetic make up, recalcitrant seeds, vegetative propagules or are threatened in their natural habitats have been well documented (Razdan and Cooking 1999). The present investigation was therefore, undertaken with the objective of establishing a reproducible micropropagation protocol for large-scale propagation of another most wanted medicinal plant *Acrous clamus* and subsequent conservation of the plant by *in vitro* culture and planting at different locations.

As appeared from the previous section *Stevia rebaudiana* is very unique plant with multipurpose uses and is a plant based non sugar sweetener. Being the most important plants and there is no report either on cultivation or on tissue culture of *Stevia rebaudiana* in Bangladesh, the present investigation was under taken with a view to establishing rapid *in vitro* mass propagation protocol of this potential new medicinal plant.

In this study, an effort was made to establish protocols for the *in vitro* mass propagation and conservation of these three important medicinal plants. The main purpose of this study was to evaluate the morphogenic potentialities of explant(s) including juvenile as well as mature tissues for most efficient production of plantlets either through precocious axillary branching or adventitious bud proliferation with the following objectives.

I. Identification and selection of suitable explant(s) for fast response and better regenerative potentialities.

II. Selection and standardization of media composition, growth regulator requirement and culture environment for consistently high production of shoots from the primary explants as well as from the sub cultured tissues.

III. To establish *in vitro* culture round the year irrespective of season for commercial exploitation and conservation.

IV. Selection of auxin type and concentration and culture condition for efficient rooting of the *in vitro* proliferated shoots.

V. Acclimatization and transplantation of *in vitro* regenerated plantlets on to soil for growing under field condition.

# CHAPTER 2

# MATERIALS AND METHODS

## 2.1 MATERIALS

### 2.1.1 Plant materials-*Aloe vera* L.

The experimental plant *Aloe vera* L. was collected from Laxmipur-Kholabaria, Natore, Bangladesh during semi-rainy season i.e. June-July, 2001. Shoot tips (**Plate 1, Fig. $A_1$**) from field grown plants were used as of primary source of explants. For further multiplication *in vitro* grown shootlets (shoot tips) were used.

### 2.1.2 Plant material-*Acorus calamus* L.

The experimental plants *Acorus calamus* L. of the family Araceae were collected from germplasm field of Bangladesh Council for Scientific and Industrial Research Laboratories, Chittagong, during rainy season, i.e. August-September, 2004. Further multiplication was done using shoot tip explants of *in vitro growth shoot cultures.* Rhizome bud shoot tips were used as the source of primary explants **(Plate 8, Fig. A).**

### 2.1.3 Plant materials-*Stevia rebaudiana* Bertoni

The seeds of *Stevia rebaudiana* were collected from USA **(Plate 13, Fig. A)**, in 2003. The seeds were germinated and seedlings were raised in pots **(Plate 13, Fig. B).** For further multiplication of shoots, nodal segments, internode and leaf segments from *in vitro* grown shoot cultures were used.

### 2.1.4 Surface sterilant and surfactant

In the present investigation mercuric chloride ($HgCl_2$) and clorox (NaOCl) were used as surface sterilizing agents while Savlon (0.3% v/v an antiseptic plus detergent, marketed in Bangladesh by ACI Bangladesh Ltd.) was used as detergent cum surfactant.

### 2.1.5 Chemicals and sources

The chemical compounds used as macro-nutrients and micro-nutrients in the present study were reagent grade (GPR) products of either Ricdel-de-Haen, Germany; BDH, England/India or E. Merck, Germany/India and Phyto Technology Laboratories ™ USA. The vitamins and growth regulators were mostly products of Phyto Technology Laboratory™ USA/E. Merck, India. A small section of them was procured from BDH, England.

### 2.1.6 Culture media

The nutrient media used in plant tissue culture are composed of several components, salts, vitamins, amino acids, growth regulators, sugars, agar or gelrite and water. All these compounds fulfill one or more functions in the *in vitro* growth of plant. The excised explants can only grow *in vitro* on a suitable artificially prepared nutrient medium which is known as culture medium. From time to time, many workers/scientists (White, Murashige & Skoog, Gambrog, Nitsch & Nitsch, Schenk, Hildebrandt and others) have proposed the composition of different nutrient media for *in vitro* growth and development of explant tissues. Mainly the MS (Murashige & Skoog 1962) medium was used in the present study to conduct most of the experiments. The medium was used either in original formulation or as in modified from to fulfill the special need of the experiments which are mentioned in appropriate places. Chemical compositions of the media are mentioned in **Appendix.**

### 2.1.7 Growth Regulators

In addition to the nutrients, it is generally necessary to add one or more growth regulators such as auxins and cytokinins to the media to support good growth of tissue and organs (Bhojwani and Razdan, 1983). The following plant growth regulators were used in the present investigation:

**Auxins:** Auxins promote cell enlargement and root initiation (Kyte, 1987); the following types were used to fulfill the experimental purpose:

Indole-3-butyric acid (IBA)

Indole-3-acetic acid (IAA)

α-napthalene acetic acid (NAA)

**Cytokinins:** Cytokinins promote cell division and shoot initiation (Kyte, 1987); the following two types were used to fulfill the experimental purpose:

6-benzyl adenine (BA)

6-furfuryl amino purine (kinetin, Kn)

**Gibberellins:** Gibberellic acid ($GA_3$)

**Natural Nutritive Fluid:** Coconut water (CW), exhibits cytokinin like activities.

### 2.1.8 Growth additives

Different brands of sucrose including common sugar and different brands of agar powder were used in the nutrient medium as carbon source and gelling agent, respectively.

## 2.2 METHODS

Aseptic technique concerning *in vitro* culture of plant tissue and organ was followed in the present study. The specific methods involved in this investigation are described under the following heads:

### 2.2.1 Preparation of culture media

The culture media used for the present investigation are stated below with their respective purposes:

a. **For shoot proliferation and multiplication:** MS (full strength of MS salts and vitamins) semi-solid medium, MS-I (½ strength of major salts but full strength of minor salts and vitamins) semi-solid medium and MS-II (½ strength of both MS salts and full strength of vitamins) semi-solid medium were used. MS-III (full strength of major salts, ½ strength of minor salts, full strength of vitamins with 200 mg/l myo-inositol and 20 mg/l thiamine) semi-solid medium were used.

b. **For root induction on *in vitro* regenerated shoots:** MS-I (½ strength of major salts but full strength of minor salts and vitamins) semi-solid medium and MS-III (full strength of major salts, ½ strength of minor salts, full strength of vitamins with 200 mg/l myo-inositol and 20 mg/l thiamine) semi-solid medium with different concentrations and combinations of auxin were used.

The first step in the reparation of any of the above culture medium was the preparation of the stock solutions. The various components of the medium were prepared into stock solutions for ready use. During the preparation of media at different concentrations, stock solutions of macro-nutrients, micro-nutrients, organic compounds and growth regulators were prepared separately as mentioned below:

**2.2.1.1 Stock solutions of macro-nutrients:** This stock solution was made up to ten times the final strength of the medium in 1000 ml of distilled water. At first ten times the weight of each salt required for one liter of the medium were weighed accurately, dissolved once at a time and sub sequentially in 750 ml of distilled water and then the final volume was made up to 1000 ml by further addition of distilled water. This stock solution was then poured into a clean glass reagent bottle and was labeled and stored in a refrigerator at 4°-6°C temperature for several weeks.

**2.2.1.2 Stock solutions of micro-nutrients:** Different types of micro-nutrients were used to prepare the MS (Murashige and Skoog, 1962) medium and its various modifications. Two stock solutions of them were prepared and stored in separate reagent bottles.

**Micro-nutrients-I:** The stock solution was made up to hundred times the final strength of the medium in 100 ml of distilled water as described for the stock solution of the macro-nutrients. All the salts (except $FeSO_4$ and $Na_2EDTA$) were weighed accurately and dissolved one at a time,

mixed them well, filtered and stored in a refrigerator at 4-6°C temperatures for several weeks.

**Micro-nutrients-2:** This stock solution was also made up to hundred times the final strength of the medium in 100 ml of distilled water. In this case two constituents $FeSO_4$ and $Na_2EDTA$ were dissolved separately in 100 ml of distilled water. The solution was heated for 24 hours at 58°C temperatures by placing in an incubator. The pH value of the solution was adjusted to 5.7 and after filtering it was stored at 4-6°C in a refrigerator.

**2.2.1.3 Stock solution of organic constitutions:** The following vitamins and amino acids were used in the present investigation for preparation of MS, MS-I, MS-II and MS-III media. The compounds were pyridoxine HCl (vitamin $B_6$), Thiamine HCl (vitamin $B_1$), Nicotinic acid (vitamin $B_3$), Myo-inositol (inositol) and Glycine (amino acid).

Each of the recommended ingredients of the organic components was made into stock solutions separately and was indicated by their respective names. Hundred times of each of the required ingredients were weighed accurately and taken in a measuring cylinder and dissolved in 100 ml of distilled water. Then the final volume was made up to 250 ml by further addition of distilled water. Then each solution was filtered, poured into a suitable glass container, labeled and stored at 4°C in a refrigerator for one month.

**2.2.1.4 Stock solution of growth regulators:** In addition to the inorganic and organic nutrients, it is generally necessary to add different growth regulators mainly auxins and cytoknins to the media for supporting suitable growth and development of tissue and organs (Bhojwani and Razdan 1983).

Stock solutions of different growth regulators were prepared separately as shown in the following table.

**Different plant growth regulators and their solvents**

| Name of the growth regulators | Amount of growth regulator taken (mg) | Solvents taken to dissolve the growth regulators (ml) | Final volume of the stock solution with distilled water (ml) | Final conc. of the growth regulator mg/ml |
|---|---|---|---|---|
| IAA | 10 | 70% KOH 1 ml | 50 | 0.2 |
| IBA | 10 | 0.1N Na OH 1ml | 50 | 0.2 |
| NAA | 10 | 0.1N Na OH 1 ml | 50 | 0.2 |
| BA | 10 | 0.1N Na OH1 ml | 50 | 0.2 |
| Kn | 10 | 0.1N Na OH1 ml | 50 | 0.2 |
| $GA_3$ | 10 | 70% KOH 1 ml | 50 | 0.2 |

To prepare the stock solution of any one of these growth regulators, 10 mg of powdered growth regulators was taken in a clean test tube and dissolved in required volume of appropriate solvent. The final volume of the solution was then made to 50 ml by adding distilled water. The solution was then poured into a 100 ml glass reagent bottle, labeled by 0.2 mg/ml and stored in a refrigerator at 4-6°C.

**2.2.1.5 Preparation of one litre culture media:** The steps as followed to make 1 litre of any of the above media (except special modifications) were based on the instruction chart given in the book of Bhojwani and Razdan (1983). The full strength of 1 litre MS medium was prepared as follows.

**Step-I.** At first 100 ml stock solution of macro-nutrients, 10 ml of each of the stock solution of micro-nutrients and 10 ml of stock solution of organic components and 30 g of sucrose were added to 500 ml distilled water and were mixed them well.

**Step-II.** Different concentrations of hormonal supplements were added either singly or in different combinations to the solution of Step-I and were mixed thoroughly.

Since each hormonal stock solution contained 10 mg the chemical in 50 ml stock solution (i.e. 0.2 mg/ml), the addition of 5 ml of any hormonal stock solution to prepare 1 litre of medium resulted in 1 mg/l concentration of that specific hormone. Similarly 0.1, 0.2, 0.5, 1.0, 2.0 and 5.0 mg/l of any hormonal supplement required addition of 0.5, 1.0, 2.5, 5.0, 10.0 and 25.0 ml amounts of specific hormaonal stock solution for 1 litre of medium.

**Step-III.** Other supplement such as coconut water was added to the medium on the basis of their expected concentrations.

**Step-IV.** The whole mixture was than made up to 1 liter by further addition of distilled water; 6-10 g agar (on the basis of its quality) was added to liter of medium.

**Step-V.** The pH of the medium was adjusted to 5.7±0.1 using either an analogue or a digital pH meter with the help of 0.1N NaOH or 0.1N HCl whichever necessary.

**Step-VI.** The mixture was then gently heated in a microwave oven with continuous stirring till complete dissolution of agar.

**Step-VII.** Fixed volume of hot medium was dispensed into culture vessels like test tubes of conical flasks. The culture vessels were plugged with absorbent cotton plugs or aluminum foil at the mouth of the culture vessels.

**Step-VIII.** Finally, the culture vessels containing medium were sterilized by autoclaving at 121°C for 20 minutes at 1.1 kg/cm$^2$ pressure. In case of flasks the medium was allowed to cool as vertically but the test tubes having media were allowed to cool as slants in case of shoot differentiation and proliferation or as vertically in case of root induction and finally stored in the culture room (not more than 5 days) for ready to use during aseptic manipulation.

Before store, test-tubes or conical flasks (culture vessels) were marked with different codes with the help of glass marker to indicate specific growth regulators supplement.

For variously modified MS medium viz. MS-I, MS-II and MS-III the above steps were also followed. But the amount of stock solution taken varied depending upon final concentration of particular ingredient in the modified medium as mentioned in the **Appendix.**

### 2.2.2 Culture techniques

The following techniques were used in the present investigation for the regeneration of complete plantlets of *Aloe vera, Acorus calamus and Stevia rebaudiana* are discussed under different headings:

**2.2.2.1 Isolation, sterilization and preparation of explants:** Healthy, disease free and desired plant parts were collected from open environment grown plants and washed thoroughly under running tap water. The plant materials were then brought to laboratory and were thoroughly washed under running tap water and placed in separate flasks. Then the materials were washed 3-4 times with distilled water and taken under running laminar airflow cabinet and transferred to 250 ml sterilized conical flask. After rinsing with 80% ethanol for 30 second they were immersed in 0.1% $HgCl_2$ for different durations of time. The materials were washed with autoclaved distilled water with at least three changes. The explants like shoot tips, nodes and leaf segments were prepared and were cultured singly in 25×150 mm culture tube containing 15-20 ml of agar gelled medium supplemented with different growth regulators.

**2.2.2.2 Maintenance and multiplication of axillary shoot proliferating cultures:** The proliferating shoot cultures established from different explants were maintained by recapturing to the freshly prepared medium for further multiplication of shoots. The cultures again produced usable shoots within few weeks of subculture. The process was repeated for several times in order to establish a continuous production of axillary or adventitious shoots.

**2.2.2.3 Induction of adventitious shoots:** Internode and leaf explants from *in vitro* grown shoots were induced for adventitious shoot regeneration on suitable media composition. The usable shoots (>3 cm) were excised from the shoot cultures and transferred them individually to the rooting media.

**2.2.2.4 Preparation and culture of microcuttings for rooting:** For rooting, the shoots with 3-4 cm in length that developed on the multiplication media were separated aseptically form the shoot masses, basal leaves were snapped off and 2-3 cm microcuttings were prepared. The individual microcutting was then transferred on the freshly prepared rooting media supplemented with different concentrations of auxins or without any auxin.

**2.2.2.5 Precautions for ensuring aseptic condition:** All inoculations and aseptic manipulations were carried out in a laminar airflow cabinet. The micro-airflow was switched on for half an hour before use and floor of the cabinet was cleaned with 80% ethyl alcohol to reduce the chances of contamination. The instruments like scalpels, forceps, needles, etc. were sterilized by an alcoholic dip and flaming method inside the laminar airflow chamber, while not in use they were kept immersed in alcohol. Other requirements like petridishes, bottles, conical flasks, cotton, distilled water, etc. were sterilized by steam sterilization method. Before the onset of inoculation hands were cleaned thoroughly by soap and then by spraying 70% ethyl alcohol. Surgical operations were carried out taking all possible care to ensure contamination free condition.

**2.2.2.6 Culture environment:** Unless mentioned specially, all cultures were maintained in an air conditioned culture room with at 16 hours photoperiod at an intensity of 2000-3000 lux (50-70 micro E. $m^{-2}$) provided by cool-white florescent tubes. The temperature of the culture room was maintained at 26±1°C but humidity was not controlled for any of the experiments.

### 2.2.3 Transplantation of plantlets under *ex vitro* environment

Plantlets with well-developed root system were taken out from the culture tubes that were kept unplugged for 4-5 days. The plantlets were washed carefully under tap water for complete removal of the medium. Polythene bags (9-15 cm) or plastic pots (9×6 cm) were kept ready filled with garden soil, organic manure and sand in the portion of 2:2:1, respectively. The soils in the polythene bags were moistened uniformly. The plantlets with small polythene bags were covered with larger (25×15 cm) polythene bags to maintain high humidity and kept them in growth chamber under artificial illumination. The potted plantlets began to form new leaves and resumed fresh growth within 5-7 days. After one week several small perforations were made on the covering bags and number of perforation was gradually increased. Within 15-20 days the covering bags were finally removed. The potted plantlets were then brought out from the growth chamber, kept under general laboratory condition and exposed to full sunlight only for 2 hours a day. In this ways the potted plantlets were successfully acclimated with natural condition through gradual increase of the duration of exposure to sunlight. They became suitable for final plantation in field after 30-40 days of acclimatization.

### 2.2.4 Computation and presentation of data

For presentation of data in the tables/figures, statistical methods were used to quantify the experimental results. Data on different parameters from different treatments of shoot proliferation were recorded after 4-10 weeks of culture and those from different experiments of rooting were recorded after 2-5 weeks of culture. Incase of shoot proliferation experiments only one parameter, percentage of explants showing proliferation, was calculated at the end of 4 weeks of culture growth and data on all other growth parameters were collected and computed at the end of the 2$^{nd}$ passage. Whereas in case of rooting experiments, excepting days to emergence of roots, data regarding other parameters were recorded after 2-5 weeks of culture.

All statistical analyses like standard error (SE) of means, analysis of various (ANOVA), Least Significant Difference (LSD) and Duncan's Multiple Range tests were performed according to standard statistical procedure (Steel and Torrie, 1982).

# CHAPTER 3

# OBSERVATION AND RESULTS

## 3.1 *Aloe vera* L.

### 3.1.1 Primary Establishment of Shoot Cultures from Shoot Tip Explants of Field Grown Plants

This part of investigation was carried out to study different aspects of *in vitro* culture of a monocot plant *Aloe vera* L. Initial experiments were collected with field grown shoot tip explants for production of micro-shoots. Further experiments were conducted with explants of *in vitro* grown shoots. Differences were observed with regard to regeneration potential and response to nutrient media. For the convenience and precise assessment, the results of different experiments of this part of investigation are described below:

In the present set of experiments, different concentrations of $HgCl_2$ were used in various durations of time. After 21 days of sterilization and incubation, it was observed that 85% of the contamination free explants sprouted. Contamination free cultures with elegant survivability were achieved by treating the explants with 0.1% $HgCl_2$ for 14 minutes. When lower concentration of $HgCl_2$ was used in shorter duration, they failed to kill the microorganisms attached to the surface of explants. On the other hand, when higher concentration of $HgCl_2$ was used in shorter duration, it showed surface sterilization of only certain percentage of explants. When the explants were treated with higher concentration of $HgCl_2$ for longer duration, it caused death of the tender tissues and the explants lost their abilities to sprout. Therefore, 14 min treatment with 0.1% $HgCl_2$ was proved to be best. After sterilization of the explants, first experiment was conducted to see the effect of three different strengths of MS medium with two different phytohormone treatments on shoot proliferation from shoot tip explants collected from field grown explants of *A.*

*vera*. Surface sterilized shoot tip explants were cultured in different treatment combinations for primary establishment of shoot cultures. These primary established shoot cultures were used for further multiplication.

In this experiment three different strength of basal nutrient medium, viz. MS, $MMS_1$ (1/2 strength major salts but full strength minor salts) and $MMS_2$ (1/2 strength major salts and minor salts) were used. In these three strengths of MS media a cytokinin either BA or KN at 0.5 mg/l concentration was added. Each treatment consisted of three replications and in each replication 10-12 explants were used. Data on percentage of explants showing shoot proliferation, number of shoots per culture and average length of the longest shoot were recorded after 42 days of culture initiation and the results obtained are shown in Table 1 and (**Plate 1, Figs. A, B** and **C**).

From the results it was observed that in all the treatments the explants formed shoots. The percentage of explants responded ranged from 20.31 to 60.13%, the number of shoots per culture ranged from 1.98 to 4.60 and the average length of the longest shoot ranged from 1.26 to 2.20 cm. The highest percentage of response 60.13% was recorded in full MS with 0.5 mg/l followed by 40.98% in $MMS_1$ with 0.5 mg/l. Number of shoots per culture was also recorded highest in the same combination. But the highest root length was recorded in $MMS_2$ with 0.5 mg/l BA followed by 1.90 cm in $MMS_1$ with BA 0.5 mg/l.

On average, out of three MS strengths highest percentage (50.34%) of explants formed shoots and maximum number (4.07) of shoots per culture were recorded in full MS but maximum shoot length (2.03 cm) was noted in $MMS_2$. From the above results it might be concluded that full MS media with BA was found to be the best combination for primary establishment of shoot cultures from shoot tip explants collected from field grown plants of *A. Vera*.

# PLATE-1

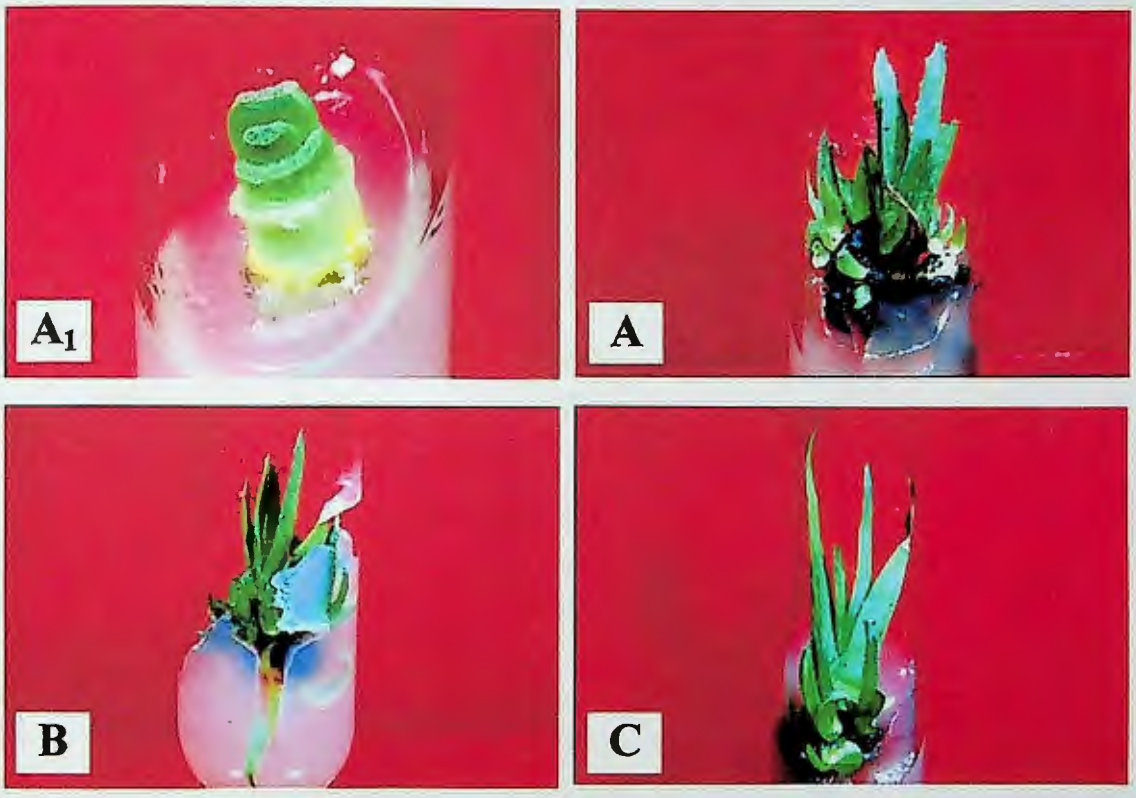



**Fig. $A_1$:** An initial shoot tip explant (*Aloe vera*) culture in *in vitro*. (from field grown mature plant).

**Fig. A:** Shoot proliferation on MS medium after 6 weeks of culture.

**Fig. B:** Shoot proliferation on $MMS_1$ medium after 6 weeks of culture.

**Fig. C:** Shoot proliferation on $MMS_2$ medium after 6 weeks of culture.

**Table 1. Effect of three different strengths of MS medium with two phytohormone treatments (0.5 mg/l Kn and 0.5 mg/l BA) on shoot proliferation from shoot tip explants collected from field grown plants of *Aloe vera*. Each treatment consisted of 3 replications and in each replication 10-12 explants were used. Data were recorded after 42 days of culture.**

| MS nutrient media in different strength | Growth regulators (mg/l) | % of explants showing proliferation | No. of shoots per culture | Average length of the longest shoot (cm) |
|---|---|---|---|---|
| MS | | 40.58 | 3.54 | 1.89 |
| $MMS_1$ | $Kn_{0.5}$ | 30.40 | 2.80 | 1.26 |
| $MMS_2$ | | 20.31 | 1.98 | 1.85 |
| MS | | 60.13 | 4.60 | 1.33 |
| $MMS_1$ | $BA_{0.5}$ | 40.98 | 3.56 | 1.90 |
| $MMS_2$ | | 30.53 | 2.92 | 2.20 |
| **Effect of MS strength[1]** | | | | |
| MS | | 50.34a | 4.07a | 1.61b |
| $MMS_1$ | | 35.69b | 3.22b | 1.58b |
| $MMS_2$ | | 25.42c | 2.45c | 2.03a |
| **Effect of phytohormone[2]** | | | | |
| Kn | | 30.43 | 2.77 | 1.67 |
| BA | | 43.88 | 3.69 | 1.81 |

[1] Means over three replications and two phytohormone treatments
[2] Means over three replications and three kinds of strength

Comparison between means of different characters was made using Duncan's multiple range test; the values in each column carrying different letters are significantly different at 5% level of probability.

### 3.1.2 Multiplication of shoots on shoot tip explants obtained from primary established shoot cultures

#### 3.1.2.1 Effect of BA or Kn singly

In this experiment effect of different concentration of BA and Kn singly on shot proliferation from *in vitro* grown shoot tip explants of *A. vera* was studied. Each treatment consisted of three replications and in each replication 10-15 explants were used. Data on percentage of explants showing shoot proliferation, number of shoots per culture and average length of the longest shoot were recorded after 35 days of culture initiation and the results obtained are shown in Table 2.

From the results it was found that in all the treatments the explants formed shoots. When BA singly was used the percentage of explants responded ranged from 25.30 to 90.91, the number of shoots per culture ranged from 1.70 to 8.81 and the average length of the longest shoot ranged from 1.25 to 4.30 cm but in case of Kn, the percentage of explants responded ranged from 20.51 to 75.93%, the number of shoots per culture ranged from 1.60 to 4.28 and the average length of the longest shoot ranged from 1.04 to 3.21cm. The highest percentage of response 90.71% was recorded in MS with 2.0 mg/l BA and 75.93% with 0.5 mg/l Kn. Number of shoots per culture was also recorded highest in the same combination. But the highest shoot length was recorded with 0.5 mg/l BA and 0.2 mg/l Kn.

Between two cytokinins BA showed the highest percentage (60.55%) of explants showing shoot proliferation. Other two characters were also recorded highest in BA. Out of seven concentrations maximum percentage of response was recorded in 2.0 mg/l followed by 1.5 mg/l. The highest number of shoots was also recorded in 2.0 mg/l but maximum shoot length was noted in 3.0 mg/l.

From the above results it might be concluded that MS media with BA was found to be the best combination for shoot proliferation from *in vitro* grown shoot tip explants of *A. Vera* and 2.0 mg/l concentration was found to be optimum.

**Table 2. Effect of different concentration of BA and Kn singly on shoot proliferation from *in vitro* grown shoot tip explants of *Aloe vera*. Each treatment consisted of 3 replications and in each replication 10-15 explants were used. Data were recorded after 35 days of culture.**

| Growth regulators (mg/l) | % of explants showing proliferation | No. of shoots per culture | Average length of the longest shoot (cm) |
|---|---|---|---|
| BA | | | |
| 0.2 | 25.30 | 1.70 | 3.60 |
| 0.5 | 40.23 | 3.54 | 4.30 |
| 1.0 | 60.42 | 4.63 | 2.20 |
| 1.5 | 85.73 | 6.85 | 1.85 |
| 2.0 | 90.91 | 8.81 | 1.25 |
| 2.5 | 75.57 | 6.09 | 1.98 |
| 3.0 | 45.66 | 3.82 | 3.31 |
| Kn | | | |
| 0.2 | 20.51 | 1.60 | 3.21 |
| 0.5 | 35.33 | 3.10 | 2.39 |
| 1.0 | 50.81 | 3.60 | 1.95 |
| 1.5 | 65.72 | 4.28 | 1.70 |
| 2.0 | 75.93 | 5.13 | 1.04 |
| 2.5 | 55.11 | 3.44 | 1.60 |
| 3.0 | 40.02 | 2.87 | 2.05 |
| **Effect of phytohormone[1](P)** | | | |
| BA | 60.55 | 5.06 | 2.64 |
| Kn | 49.06 | 3.43 | 1.99 |
| **Effect of concentration[2](C)** | | | |
| 0.2 mg/l | 22.90 | 1.65 | 3.76 |
| 0.5 mg/l | 37.78 | 3.32 | 2.00 |
| 1.0 mg/l | 55.61 | 4.12 | 2.08 |
| 1.5 mg/l | 75.73 | 5.57 | 1.78 |
| 2.0 mg/l | 83.01 | 6.97 | 1.15 |
| 2.5 mg/l | 65.34 | 4.77 | 1.79 |
| 3.0 mg/l | 42.84 | 3.3 | 2.68 |
| **LSD at 5% for P** | **7.00** | **1.20** | **NS** |
| **LSD at 5% for C** | **5.45** | **0.94** | **1.00** |
| **LSD at 5% for P×C** | **8.56** | **1.76** | **1.23** |

[1] Means over three replications and seven kinds of concentration

[2] Means over three replications and two kinds of phytohormone treatments

### 3.1.2.2 Effect of BA in combination with Kn

Effect of different concentration and combination of BA with Kn on shot proliferation from *in vitro* grown shoot tip explants of *A. vera* was studied in this experiment. Each treatment consisted of three replications and in each replication 8-12 explants were used. Data on percentage of explants showing shoot proliferation, number of shoots per culture and average length of the longest shoot were recorded after 35 days of culture initiation and the results obtained are shown in Table 3.

From the results it was found that in all the treatments the explants formed shoots. The percentage of explants responded ranged from 40.23 to 90.51%, the number of shoots per culture ranged from 4.09 to 10.46 and the average length of the longest shoot ranged from 1.96 to 3.32 cm.

Out of fifteen different combinations of BA with Kin the highest percentage (90.51%) of explants showing shoot proliferation and the maximum number of shoot per culture (10.46) were recorded in 2.0 mg/l BA+0.5 mg/l Kn. But maximum average length of longest shoot was recorded in MS with 1.5 mg/l BA+0.5 mg/l Kn (**Plate 2, Fig. A**).

From the above results it might be concluded that the best combination for shoot proliferation from *in vitro* grown shoot tip explants of *A. Vera* was 2.0 mg/l BA+0.5 mg/l Kn (**Plate 2, Fig. B**).

**Table 3. Effect of different concentration and combination of BA with Kn on shoot proliferation from *in vitro* grown shoot tip explants of *A. vera*. Each treatment consisted of 3 replications and in each replication 8-12 explants were used. Data were recorded after 35 days of culture.**

| Growth regulators (mg/l) | % of explants showing proliferation | No. of shoots per culture | Average length of the longest shoot (cm) |
|---|---|---|---|
| BA + Kn | | | |
| 0.5 + 0.2 | 45.31 | 4.12 | 3.18 |
| 0.5 + 0.5 | 50.89 | 4.30 | 2.33 |
| 0.5 + 1.0 | 45.92 | 4.21 | 3.09 |
| 1.0 + 0.2 | 60.20 | 5.25 | 2.45 |
| 1.0 + 0.5 | 65.03 | 5.41 | 2.86 |
| 1.0 + 1.0 | 55.81 | 4.76 | 2.67 |
| 1.5 + 0.2 | 70.32 | 6.23 | 2.54 |
| 1.5 + 0.5 | 75.51 | 6.92 | 3.32 |
| 1.5 + 1.0 | 60.74 | 5.39 | 3.03 |
| 2.0 + 0.2 | 80.15 | 9.18 | 2.44 |
| 2.0 + 0.5 | 90.51 | 10.46 | 1.96 |
| 2.0 + 1.0 | 65.02 | 5.94 | 2.81 |
| 2.5 + 0.2 | 55.93 | 4.93 | 3.69 |
| 2.5 + 0.5 | 50.32 | 4.40 | 2.09 |
| 2.5 + 1.0 | 40.23 | 4.09 | 2.39 |
| LSD at 5% level | **6.62** | **1.23** | **0.80** |

# PLATE-2



**Fig. A:** Proliferation of shoots on MS + 1.5 mg/l BA+0.5 mg/l Kn after 6 weeks of culture.

**Fig. B:** Proliferation of shoots on MS + 2.0 mg/l BA+0.5 mg/l Kn after 6 weeks of culture.

### 3.1.2.3 Effect of BA in combination with NAA or IAA

In this experiment effect of different concentrations of BA with NAA or IAA on shot proliferation from *in vitro* grown shoot tip explants of *A. vera* was studied. Each treatment consisted of three replications and in each replication 10-12 explants were used. Data on percentage of explants showing proliferation, number of shoots per culture and average length of the longest shoot were recorded after 42 days of culture initiation and the results obtained are shown in Table 4 and **Plate 3 (Figs. A, B and C).**

From the results it was found that in all the treatments the explants formed shoots and slight to profuse callus. When BA with NAA were used the percentage of explants responded ranged from 50.53 to 95.30%, the number of shoots per culture ranged from 5.13 to 12.43 and the average length of the longest shoot ranged from 2.62 to 4.92 cm but in case of BA+IAA, the percentage of explants responded ranged from 30.15 to 60.42, the number of shoots per culture ranged from 3.32 to 6.57 and the average length of the longest shoot ranged from 1.81 to 2.85 cm. The highest percentage of response 95.30% was recorded in MS with 2.0 mg/l BA+0.2 mg/l NAA followed by 80.58 with 2.5 mg/l BA+0.2 mg/l NAA. Number of shoots per culture was also recorded highest in the same combination. But the highest shoot length was recorded in 2.0 mg/l BA+0.5 mg/l NAA.

On average between two combinations BA plus NAA showed the highest percentage of explants showing proliferation. Average number of shoot per culture and average length of longest shoot were also recorded highest in this combination.

So, from the above results it might be concluded that the combination of 2.0 mg/l BA+0.2 mg/l NAA was found to be the best combination for shoot proliferation from *in vitro* grown shoot tip explants of *A. Vera.*

**Table 4. Effect of different concentration and combination of BA with NAA, IBA or IAA on shoot proliferation from *in vitro* grown shoot culture of *A. vera*. Each treatment consisted of 3 replications and in each replication 10-12 explants were used. Data were recorded after 42 days of culture.**

| Growth regulators (mg/l) | % of explants showing proliferation | No. of shoots per culture | Average length of the longest shoot (cm) | Callus formation |
|---|---|---|---|---|
| BA + NAA | | | | |
| 1.0 + 0.1 | 70.23 | 7.41 | 3.12 | + |
| 1.0 + 0.2 | 75.84 | 8.18 | 2.62 | ++ |
| 1.0 + 0.5 | 50.53 | 5.13 | 3.41 | +++ |
| 2.0 + 0.1 | 80.02 | 9.76 | 3.93 | ++ |
| 2.0 + 0.2 | 95.30 | 12.43 | 3.01 | ++ |
| 2.0 + 0.5 | 70.81 | 7.15 | 4.92 | +++ |
| 2.5 + 0.1 | 75.21 | 8.37 | 3.32 | + |
| 2.5 + 0.2 | 80.58 | 9.59 | 2.91 | + |
| 2.5 + 0.5 | 60.11 | 5.88 | 3.72 | ++ |
| Mean | **73.18a** | **7.40a** | **3.44a** | |
| BA + IAA | | | | |
| 1.0 + 0.1 | 45.52 | 4.52 | 2.59 | + |
| 1.0 + 0.2 | 50.93 | 5.14 | 2.03 | + |
| 1.0 + 0.5 | 35.12 | 3.82 | 2.38 | ++ |
| 2.0 + 0.1 | 40.18 | 4.23 | 2.12 | + |
| 2.0 + 0.2 | 55.22 | 5.36 | 2.67 | + |
| 2.0 + 0.5 | 60.42 | 6.57 | 2.85 | ++ |
| 2.5 + 0.1 | 30.15 | 3.32 | 1.81 | + |
| 2.5 + 0.2 | 35.03 | 3.92 | 2.43 | + |
| 2.5 + 0.5 | 50.09 | 4.74 | 2.00 | + |
| **Mean** | **44.74c** | **4.62c** | **2.32c** | |

+, ++, +++ indicate slight, considerable and profuse callusing, respectively.

Comparison between means of different characters was made using Duncan's multiple range test; the values in each column carrying different letters are significantly different at 5% level of probability.

# PLATE-3

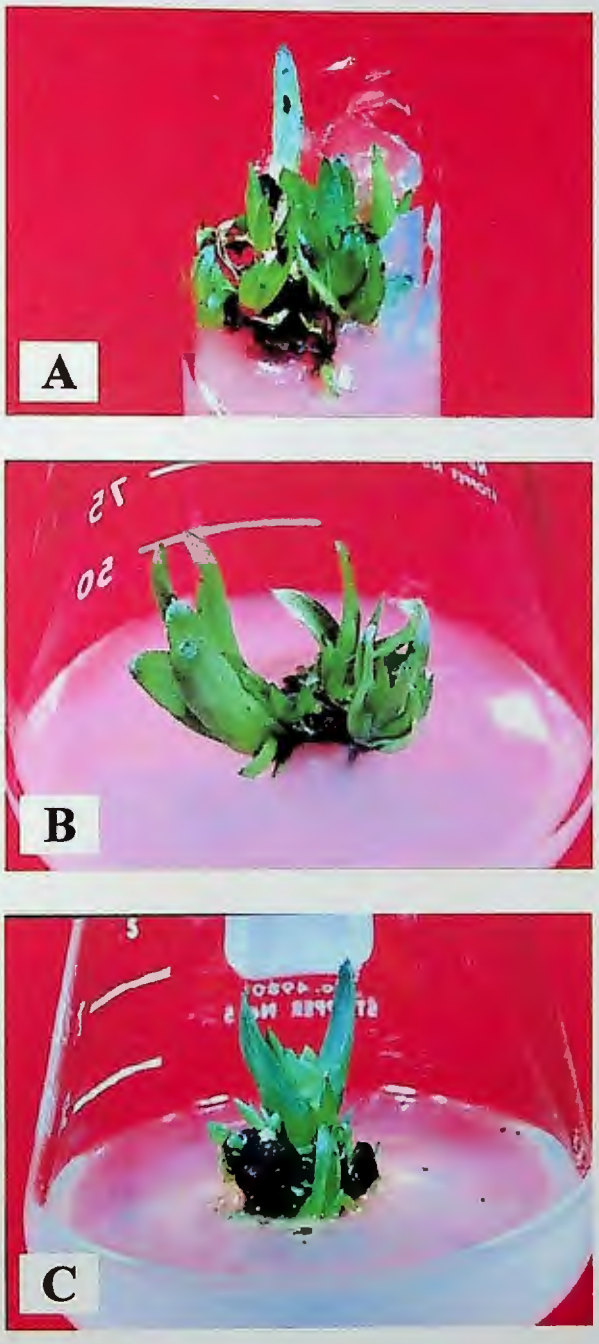



**Fig. A:** Axillary shoot proliferation on MS+ 2.0 mg/l BA+0.2 mg/l NAA after 6 weeks of culture.

**Fig. B:** Axillary shoot proliferation on MS+ 2.5 mg/l BA+0.2 mg/l NAA after 6 weeks of culture.

**Fig. C:** Axillary shoot proliferation on MS+ 2.0 mg/l BA + 0.5 mg/l IAA after 4 weeks of culture.

### 3.1.2.4 Effect of BA in combination with Kn and NAA

In this experiment effect of three different concentration and combination of BA plus Kn and NAA on shoot proliferation from *in vitro* grown shoot tip explants of *A. vera* was studied. Each treatment consisted of three replications and in each replication 10-12 explants were used. Data on percentage of explants showing shoot proliferation, number of shoots per culture and average length of the longest shoot were recorded after 42 days of culture initiation and the results obtained are shown in Table 5 and **Plate 4** (**Figs. A** and **B**).

From the results it was found that in all the treatments the explants formed shoots. The percentage of explants responded ranged from 55.56 to 98.96, the number of shoots per culture ranged from 5.13 to 15.39 and the average length of the longest shoot ranged from 1.50 to 3.87 cm.

Out of twelve combinations of BA plus Kn and NAA the highest percentage (98.96%) of explants showing shoot proliferation and maximum number of shoot per culture (15.39) were recorded in MS with 2.0 mg/l BA+0.5 mg/l Kn+0.2 mg/l NAA. Callus formation at the cut surface of the cultured explants was observed in some treatments.

From the above results it might be concluded that the best combination for shoot proliferation from *in vitro* grown shoot tip explants of *A. Vera* was 2.0 mg/l BA+0.5 mg/l Kn+0.2 mg/l NAA.

**Table 5. Effect of different concentration and combination of BA with Kn and NAA on shoot proliferation from *in vitro* grown shoot tip explants *A. vera*. Each treatment consisted of 3 replications and in each replication 10-12 explants were used. Data were recorded after 42 days of culture.**

| Growth regulators (mg/l) | % of explants showing proliferation | No. of shoots per culture | Average length of the longest shoot (cm) | Callus formation |
|---|---|---|---|---|
| BA+Kn+NAA | | | | |
| 1.0+0.2+0.1 | 65.63 | 6.35 | 3.01 | - |
| 1.0+0.5+0.1 | 70.86 | 7.69 | 2.39 | - |
| 1.0+0.2+0.2 | 55.56 | 5.13 | 3.87 | + |
| 1.0+0.5+0.2 | 75.01 | 8.70 | 2.60 | ++ |
| 2.0+0.2+0.1 | 80.90 | 10.21 | 2.59 | - |
| 2.0+0.5+0.1 | 95.77 | 12.85 | 2.60 | - |
| 2.0+0.2+0.2 | 70.31 | 7.88 | 2.95 | ++ |
| 2.0+0.5+0.2 | 98.96 | 15.39 | 1.50 | + |
| 2.5+0.2+0.1 | 70.30 | 7.52 | 3.11 | - |
| 2.5+0.5+0.1 | 55.99 | 5.33 | 3.20 | - |
| 2.5+0.2+0.2 | 65.13 | 6.10 | 3.10 | + |
| 2.5+0.5+0.2 | 75.44 | 8.58 | 2.99 | ++ |
| **LSD at 5% level** | **6.14** | **1.09** | **0.42** | |

**-, +, ++ indicate no, slight and considerable callusing, respectively.**

# PLATE-4

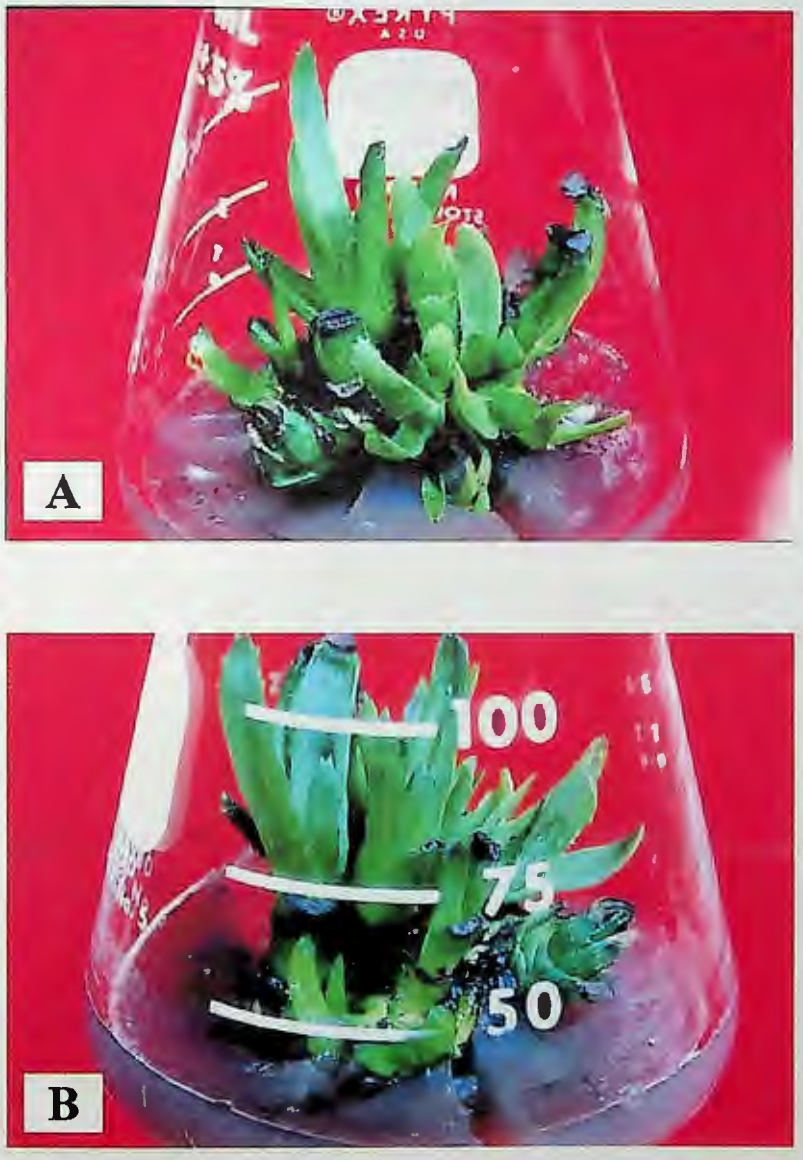
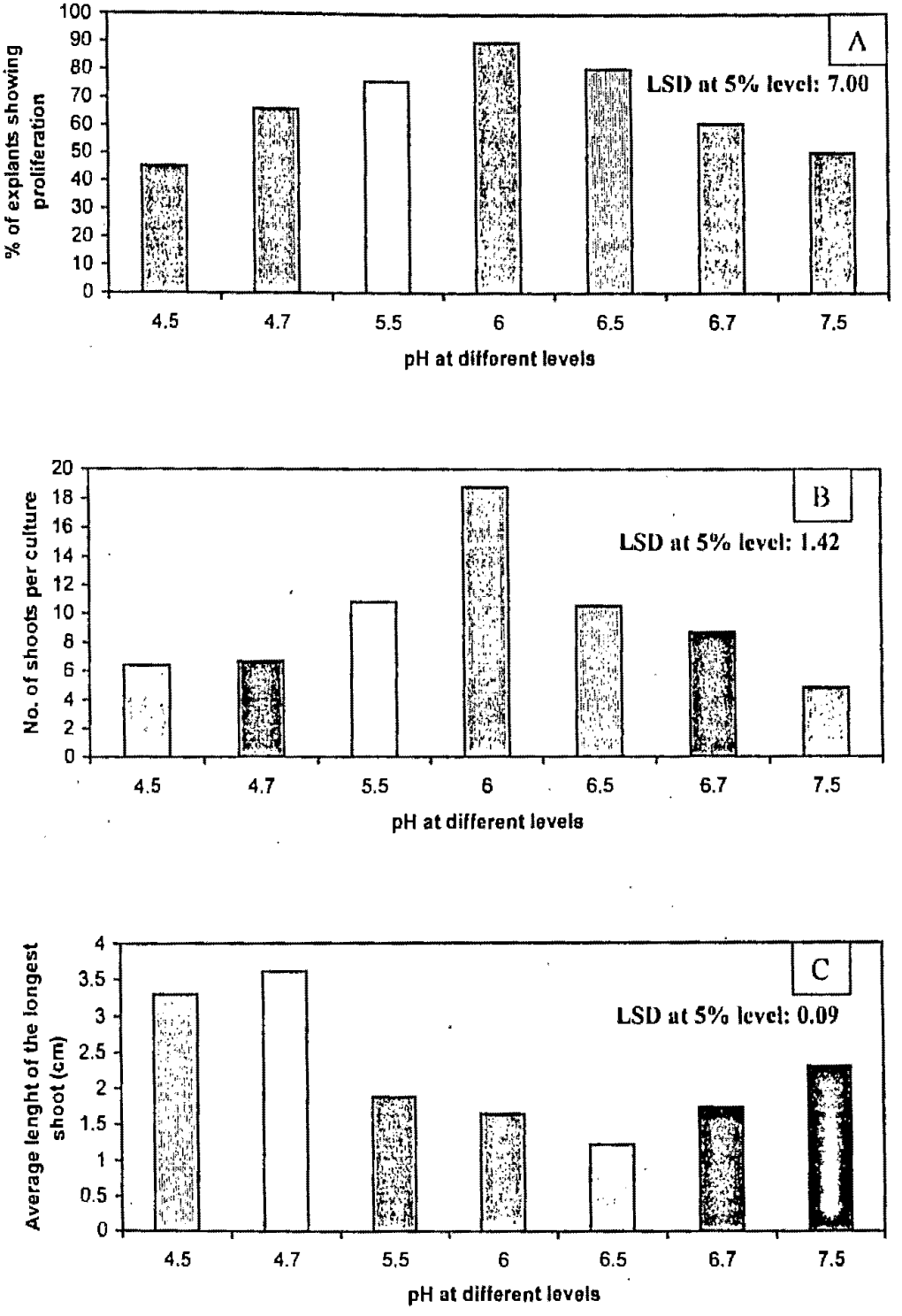



**Fig. A:** A cluster of axillary shoots proliferation on MS + 2.0 mg/l BA+0.5 mg/l Kn+0.2 mg/l NAA after 6 weeks of culture.

**Fig. B:** Rapidly growing a cluster of axillary shoot proliferation on MS + 2.0 mg/l BA + 0.5 mg/l Kn + 0.1 mg/l NAA after 5 weeks of culture.

### 3.1.2.5 Effect of different pH levels

pH means negative logarithm of hydrogen ion concentration in a solution. In this experiment the explants were cultured on MS medium having 2.0 mg/l BA + 0.5mg/l Kn + 0.2mg/l NAA at seven different pH levels viz. 4.5, 4.7, 5.5, 6.0, 6.5, 6.7 and 7.5. Media with these levels of pH were used to standardize the optimum pH value for MS medium for *in vitro* shoot proliferation of *Aloe vera*. Percentage of proliferated cultures, number of shoots per culture and average length of the longest shoots were recorded after four weeks of culture. At pH 4.5 and 4.7, filter paper bridges were used, as the media did not form gel at lower pH values. The highest percentage of explants showing shoot proliferation was recorded as 90% at the medium having the pH value of 6.0 and lowest percentage of explants showing shoot proliferation was recorded as 45% at the pH value of 4.5. The highest number of shoots per culture was recorded in medium having pH value 6.0 and the number of shoots per culture was counted as 18.8. The lowest number of shoots per culture 6.4 was counted at pH level 4.5 Highest average length of the longest shoot per culture was 3.6 cm recorded in medium having pH value 4.7. At pH 6.5 the lowest length of shoot was measured as 1.2 cm. It is evident from the results that the pH level not only affected the frequency of cultures showing shoot proliferation but also collected the number and growth of the proliferated shoots. It also reveals that the *Aloe vera* shoots favour slightly acidic pH medium (**Graph 1, Plate 5, Fig. A**).




**Graph 1.** Effect of different pH levels of culture media on shoot proliferation in *A. vera*. A. Percentage of explants showing shoot proliferation, B. Number of shoots per culture C. Average length of the longest shoot (cm).

### 3.1.2.6 Effect of different brands and levels of agar powder

In this experiment effect of different types (Merck Ltd., Mumbai, India; *Phyto* Technology Laboratories, USA; BDH chemicals Ltd. Poole, England, UK) and strengths of agar powder on shoot proliferation from *in vitro* grown shoot tip explants of *A. vera* on MS medium supplemented with BA 2.0 mg/l + Kn 0.5 mg/l + NAA 0.2 mg/l was studied. Each treatment consisted of three replications and in each replication 10-15 explants were used. Data on percentage of explants showing shoot proliferation, number of shoots per culture and average length of the longest shoot were recorded after 35 days of culture initiation and the results obtained are shown in Table 6 and **Plate 5 (Fig. B)**.

From the results it was found that in all the treatments the explants formed shoots except in 10 gm/l agar. The percentage of explants responded ranged from 0 to 90.54%, 0 to 95.32% and 0 to 95.56% in case of Merck (India), *Phyto* Technolgy (USA) and BDH (UK) respectively. The number of shoots per culture ranged from 0 to 11.73, 0 to 13.83 and 0 to 15.23 in Merck, *Phyto* Technolgy and BDH respectively.

In each type of agar brand five strengths were used. Out of five strengths of different agar brand, the highest percentage of explants showing shoot proliferation was noticed 90.54% in Merck Ltd., Mumbai, India; 95.32% in *Phyto* Technology Laboratories, USA and 95.56% in BDH Chemicals Ltd. Poole, England, UK and in all cases the best results were observed with 7 g/l of agar.

From the above results it might be concluded that the best combination for shoot proliferation from *in vitro* grown shoot tip explants of *A. Vera* was 7 g/l agar and no significant differences were noticed among the three brands of agar used.

**Table 6. Effect of different types and strengths of agar powder on shoot proliferation from *in vitro* grown shoot tip explants on MS medium supplemented with BA 2.0 mg/l + Kn 0.5 mg/l + NAA 0.2 mg/l. Each treatment consisted of 3 replications and in each replication 10-15 explants were used. Data were recorded after 35 days of culture.**

| Different types of agar brand | Agar at different strengths (g/l) | % of explants showing proliferation | No. of shoots per culture | Average length of the longest shoot (cm) |
|---|---|---|---|---|
| Merck Ltd. Mumbai, India | 6 | 70.32 c | 9.81 b | 3.19 bc |
| | 7 | 90.54 a | 11.73 a | 3.93 a |
| | 8 | 80.65 b | 11.14 a | 3.48 ab |
| | 9 | 55. 66 d | 7.35 c | 2.82 c |
| | 10 | - | - | - |
| **Mean** | | **74.29a** | **10.01a** | **3.36a** |
| *Phyto* Technology™ Laboratories USA | 6 | 80.65 b | 11.27 b | 3.54 b |
| | 7 | 95.32 a | 13.83 a | 4.33 a |
| | 8 | 70.21 c | 11.09 b | 3.28 b |
| | 9 | 50.35. d | 7.23 c | 2.91 b |
| | 10 | - | - | - |
| **Mean** | | **74.13a** | **10.86a** | **3.52a** |
| BDH chemicals Ltd. Poole, England, UK | 6 | 85.98 b | 11.41 b | 3.87 b |
| | 7 | 95.56 a | 15.23 a | 4.53 a |
| | 8 | 65.67 c | 9.87 c | 3.58 bc |
| | 9 | 45.81 d | 7.09 d | 3.03 c |
| | 10 | - | - | - |
| **Mean** | | **73.24a** | **10.90a** | **3.75a** |

Comparison of the mean values for different characters was made using Duncan's multiple range test; the values in each column carrying same letters are not significantly different at 5% level of probability.

# PLATE-5

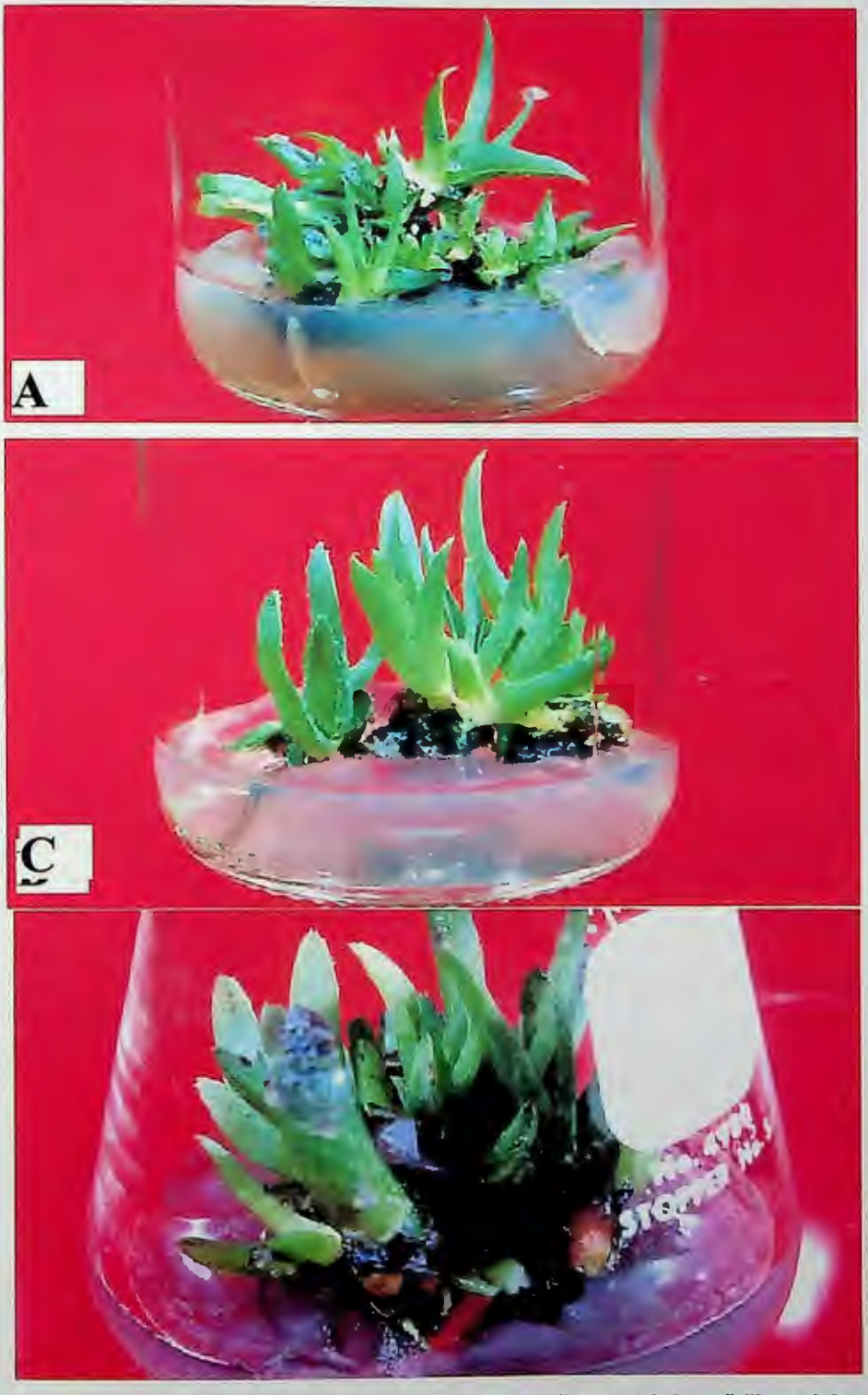



**Fig. A:** Effect of pH for mas propagation on MS + 2.0 mg/l BA + 0.5 mg/l Kn + 0.2 mg/l NAA adjusted at 6.0 level of pH, after 6 weeks.

**Fig. B:** Effect of agar for mass propagation on MS + 2.0 mg/l BA + 0.5 mg/l Kn + 0.2 mg/l NAA with 7.0 mg/l agar after 3 weeks.

**Fig. C:** Effect of sucrose for mass propagation on MS + 2.0 mg/l BA + 0.5 mg/l Kn + 0.2 mg/l NAA with 40 g/l local market sugar, after 4 weeks of growth.

### 3.1.2.7 Effect of different types and levels of sucrose

In this experiment effect of different types (Merck Ltd., Mumbai, India; *Phyto* Technology Laboratories, USA; BDH Chemicals Ltd., England and local market sugar) and concentration (20, 30, 40, 50 g/l) of sucrose on shoot proliferation from *in vitro* grown shootlet explants of *A. vera* in MS medium containing BA 2.0 mg/l + Kn 0.5 mg/l + NAA 0.2 mg/l was studied. Each treatment consisted of three replications and in each replication 10-15 explants were used. Data on percentage of explants showing shoot proliferation, number of shoots per culture and average length of the longest shoot were recorded after 28 days of culture initiation and the results obtained are shown in Table 7 and **Plate 5 (Fig. C)**.

From the results it was found that in all the treatments the explants formed shoots. The percentage of explants responded ranged from 75.41 to 85.05%, 70.92 to 99.02%, and 65.45 to 99.22% and 70.02 to 82.71% in case of Merck Ltd. (India), *Phyto* Technolgy (USA) and BDH (UK) and local market sugar respectively. The number of shoots per culture ranged from 9.57 to 15.61, 9.27 to 18.35, 9.05 to 18.68 and 9.36 to 14.37 in Merck Ltd. (India), *Phyto* Technolgy (USA) and BDH (UK) and local market respectively.

In each type of sucrose four levels were used. Out of four levels of sucrose, the highest percentage of explants showing shoot proliferation was recorded 85.05% in Merck Ltd., Mumbai; 99.02% in *Phyto* Technology Laboratories, USA, 99.22% in BDH Chemicals Ltd., England and 82.71% in local market sugar. Except local market sugar in all cases the best results were observed in 30 g/l of sucrose and in case of local market 40 g/l was found best.

Among the four brands on average Phyto USA brand was found to be the best and among the four concentrations on average 30g/l sucrose was recorded to be the best.

From the above results it might be concluded that 30 g/l sucrose of BDH Chemicals Ltd., England showed the best results for shoot proliferation from *in vitro* grown shoot tip explants of *A. Vera* with 2.0 mg/l BA+0.5 mg/l Kn+0.2 mg/l NAA.

**Table 7. Effect of different types and concentration of sucrose on shoots proliferation from *in vitro* grown shootlet explant on MS medium containing BA 2.0 mg/l + Kn 0.5 mg/l + NAA 0.2 mg/l. Each treatment consisted of 3 replications and in each replication 10-15 explants were used. Data were recorded after 28 days of culture.**

| Different brands of sucrose | Sucrose at different levels (g/l) | % of explants showing proliferation | No. of shoots per culture | Average length of the longest shoot (cm) |
|---|---|---|---|---|
| Merck Ltd. Mumbai | 20 | 75.41 | 9.63 | 3.26 |
| | 30 | 85.05 | 15.61 | 2.98 |
| | 40 | 80.13 | 9.82 | 3.45 |
| | 50 | 75.84 | 9.57 | 3.53 |
| *Phyto* Technology Laboratories™, USA | 20 | 80.22 | 11.09 | 3.44 |
| | 30 | 99.02 | 18.35 | 3.11 |
| | 40 | 85.11 | 11.36 | 3.61 |
| | 50 | 70.92 | 9.27 | 3.22 |
| BDH Chemicals Ltd. England | 20 | 85.88 | 11.43 | 3.67 |
| | 30 | 99.22 | 18.68 | 3.06 |
| | 40 | 87.03 | 11.59 | 3.89 |
| | 50 | 65.45 | 9.05 | 3.48 |
| Local Market Sugar | 20 | 70.02 | 9.48 | 3.14 |
| | 30 | 78.13 | 11.13 | 3.51 |
| | 40 | 82.71 | 14.37 | 2.75 |
| | 50 | 75.85 | 9.36 | 3.37 |
| Effect of Brand[1] | | | | |
| **Merck Ltd. Mumbai** | | 79.11a | 11.16b | 3.31a |
| ***Phyto* Technology aboratories™, USA** | | 83.82a | 12.52a | 3.34a |
| **BDH Chemicals Ltd. England** | | 84.39a | 12.69a | 3.53a |
| **Local Market Sugar** | | 76.68b | 11.08b | 3.19a |
| **Effect of concentration[2]** | | | | |
| 20 g/l | | 77.88c | 10.41c | 3.38a |
| 30 g/l | | 90.36a | 16.19a | 3.17a |
| 40 g/l | | 83.75b | 11.79b | 3.43a |
| 50 g/l | | 72.02c | 9.31d | 3.40a |

[1] Means over three replications and four kinds of concentration
[2] Means over three replications and four brands of sucrose

Comparison between means of different characters was made using Duncan's multiple range test; the values in each column carrying different letters are significantly different at 5% level of probability.

### 3.1.3 Rooting of *in vitro* proliferated shoots

In this experiment effect of different concentration of single auxin (NAA, IBA or IAA) in $MMS_1$ medium for induction of root from *in vitro* grown microshoots of *A. vera* was studied. Each treatment consisted of three replications and in each replication 10-15 explants were used. Data on percentage of explants showing proliferation, number of shoots per culture and average length of the longest shoot were recorded after 7-8 weeks of culture initiation and the results obtained are shown in Table 8 and **Plate 6 (Figs. A, B** and **C)**.

From the results it was found that in the auxin omitted $MMS_1$ failed to induce any root formation. In three concentrations of IAA root induction was also not observed. When NAA was used the percentage of explants responded ranged from 40.36 to 80.25%, the number of root per microshoot ranged from 2.98 to 6.71 and the average length of the longest root ranged from 2.05 to 2.71 cm but when IBA was used the percentage of explants responded ranged from 20.17 to 60.85%, the number of root per microshoot ranged from 1.78 to 4.51 and the average length of the longest root ranged from 1.02 to 2.33 cm. When IAA was used rooting was observed only in medium with 1.0 mg/l where the percentage of explants responded was 10.36%, the number of roots per microshoot was 1.09 and length of the longest root was 0.56 cm. Among the twelve/treatments the highest percentage of response 80.25% was recorded in MS with 0.2 mg/l NAA followed by 60.85 with 0.2 mg/l IBA. Number of roots per microshoot and length of the longest root were also recorded highest in $MMS_1$ with 0.2 mg/l NAA.

On average, among different concentrations of single auxin the highest percentage of explants showing root proliferation was recorded 57.74%, 38.14% and 10.36% in NAA, IBA and IAA respectively.

From the above results it might be concluded that $MMS_1$ media with single NAA at a concentration of 0.2 mg/l was found to be the best treatment for root proliferation from *in vitro* grown microshoots of *A. Vera*.

**Table 8. Effect of different concentration of single auxin in MMS1 medium for induction of roots from *in vitro* grown micro shoots. Each treatment consisted of 3 replications and in each replication 10-15 explants were used. Data were recorded after 7-8 weeks of culture.**

| Different type of auxins | Different conc. of single auxin (mg/l) | % of micro shoots rooted | No. of root per micro-shoots | Average length of roots (cm) |
|---|---|---|---|---|
| Nil | - | - | - | - |
| NAA | 0.1 | 40.36 | 2.98 | 2.05 |
| | 0.2 | 80.25 | 6.71 | 2.71 |
| | 0.5 | 60.24 | 4.91 | 2.55 |
| | 1.0 | 50.12 | 3.88 | 2.11 |
| **Mean** | | **57.74a** | **4.62a** | **2.35a** |
| IBA | 0.1 | 30.91 | 2.77 | 1.66 |
| | 0.2 | 60.85 | 4.51 | 2.33 |
| | 0.5 | 40.64 | 2.75 | 1.80 |
| | 1.0 | 20.17 | 1.78 | 1.02 |
| **Mean** | | **38.14b** | **2.45b** | **1.70b** |
| IAA | 0.1 | - | - | - |
| | 0.2 | - | - | - |
| | 0.5 | - | - | - |
| | 1.0 | 10.36 | 1.09 | 0.56 |
| **Mean** | | **10.36c** | **1.09c** | **0.56c** |

- Indicates no response.

# PLATE-6




**Fig. A:** Rooting on MS medium + 0.2 mg/l IBA

**Fig. B:** Rooting on $MMS_1$ medium + 0.5 mg/l NAA

**Fig. C:** Rooting on $MMS_1$ medium + 0.2 mg/l NAA

### 3.1.4 Acclimatization and establishment of *in vitro* regenerated plantlets on soil

After rooting of the *in vitro* regenerated microshoots they were transferred to soil under *ex vitro* condition. Prior to transplantation the individual regenerated plantlet was brought out of the culture medium and its root system was made agar-gel free by continuous flashing of running tap water. Then the plantlets were transferred to small earthen pot (**Plate 7, Fig. A**) containing sun sterilized garden soil, sand and compost (2: 1: 1) and gradually acclimatized to out door conditions. The potted plants were then watered one time daily and covered with perforated polyethylene bag and kept in a room for 7-10 days. After a few days of indoor acclimatization the plantlets from small pots were transplanted directly to the larger pots and kept in outdoor condition. Plantlets were initially established in small pots with a view to their easy handling during transplantation. It was noticed that the prevailing atmospheric conditions (mostly humidity and temperature) of the transplanting season very much influenced the initial survival of potted plantlets. However 70% of *Aloe vera* plantlets were established under *ex vitro* conditions (**Plate 7, Fig. B**) when they were transferred on garden soil and humus.

# PLATE-7



**Fig. A:** Regenerated plantlets of *Aloe vera* to small earthen pots containing soil + sand + compost (2:1:1) after 5 weeks.

**Fig. B:** Under *ex vitro* condition with garden soil and humus, after 10 weeks.

## 3.2. *A. calamus*

### 3.2.1 Primary establishment of shoot cultures from rhizome tips

In *A. calamus* rhizome tips from growing plants were used as primary explants. Rhizomes were surface sterilized using different concentrations of $HgCl_2$ for different duration and 15 minutes treatments with 0.1 mg/l $HgCl_2$ solution was found to be optimum where 80 – 85% of cultured explants survived and formed shoots. The first experiment was conducted to see the effect of different concentration of BA and Kn singly on shoot proliferation from rhizome tip explants collected from rhizomes of *A. calamus* on MS medium was studied. Each treatment consisted of three replications and in each replication 10-12 explants were used. Data on percentage of explants showing shoot proliferation, number of shoots per culture and average length of the longest shoot were recorded after 6 weeks of culture initiation and the results obtained are shown in Table 9 and **Plate 8 (Figs. A, B** and **C)**.

From the results it was found that in all the treatments the explants formed shoots. When BA was used the percentage of explants responded ranged from 60.20 to 95.86%, the number of shoots per culture ranged from 3.92 to 6.00 and the average length of the longest shoot ranged from 2.38 to 3.31 cm. In case of Kn, the percentage of explants responded ranges from 65.39 to 90.11%, the number of shoots per culture ranged from 2.91 to 4.85 and the average length of the longest shoot ranged from 2.19 to 3.15 cm. Among the treatments the highest percentage of response 95.86% was recorded in 3.0 mg/l BA. But the number (6.00) of shoots per culture and highest shoot length (3.31 cm) were recorded in 2.0 mg/l BA.

On average, between two cytokinins BA was found superior than Kn in all the three characters. Among five concentrations on average 2.0 mg/l was found optimum.

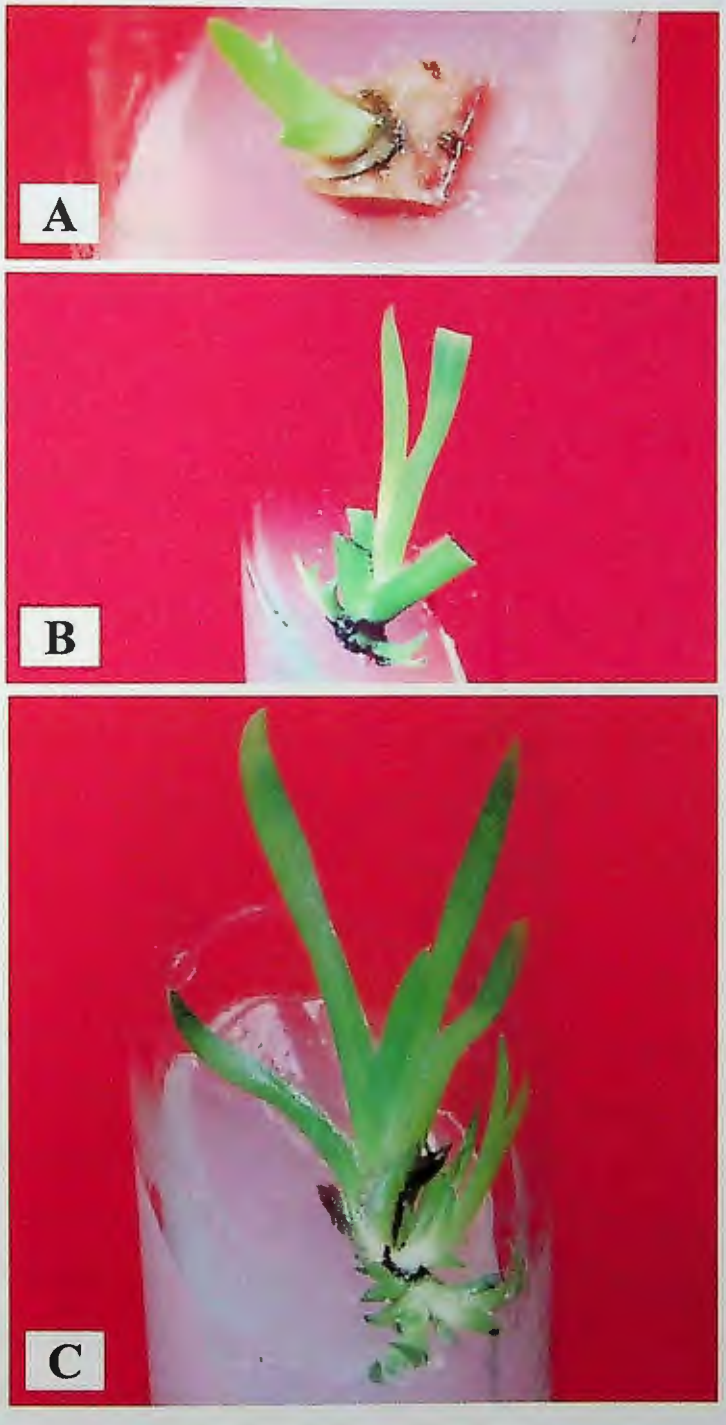
From the above results it might be concluded that MS media with 3.0 mg/l concentration of BA was found to be the best for shoot proliferation from rhizome tip explants of field growing plants of *A. calamus*.

**Table 9. Effect of different concentration of BA and Kn singly on shoot proliferation from rhizome tips of growing plants of *A. calamus* on MS medium. Each treatment consisted of 3 replications and in each replication 10-12 explants were used. Data were recorded after 6 weeks of culture.**

| Growth regulators (mg/l) | % of explants showing proliferation | No. of shoots per culture | Average length of the longest shoot (cm) |
|---|---|---|---|
| BA | | | |
| 0.5 | 70.03 | 4.43 | 2.49 |
| 1.0 | 90.81 | 5.91 | 3.09 |
| 2.0 | 90.56 | 6.00 | 3.31 |
| 3.0 | 95.86 | 5.15 | 2.85 |
| 4.0 | 60.20 | 3.92 | 2.38 |
| Kn | | | |
| 0.5 | 75.51 | 3.60 | 2.23 |
| 1.0 | 85.39 | 3.99 | 2.71 |
| 2.0 | 90.11 | 4.85 | 3.15 |
| 3.0 | 80.07 | 3.43 | 2.47 |
| 4.0 | 65.39 | 2.91 | 2.19 |
| **Effect of phytohormone[1]** | | | |
| BA | 83.29 | 5.12 | 2.82 |
| Kn | 79.29 | 3.76 | 2.55 |
| **LSD at 5% level** | **6.71** | **1.13** | **NS** |
| **Effect of concentration** | | | |
| 0.5 mg/l | 72.77 | 4.02 | 3.23 |
| 1.0 mg/l | 88.10 | 4.95 | 2.90 |
| 2.0 mg/l | 94.84 | 5.43 | 2.36 |
| 3.0 mg/l | 87.97 | 4.29 | 2.29 |
| 4.0 mg/l | 62.80 | 3.42 | 2.66 |
| **LSD at 5% level** | **5.11** | **0.99** | **NS** |

[1]Means over three replications and five phytohormone concentrations
[2] Means over three replications and two kinds of phytohormone

# PLATE-8




Shoot proliferation from Rhizome tip explants of field grown *Acorus calamus* plant cultured on MS medium with 2.0 mg/l BA (**Fig. A, B and C**) after 2 weeks, 4 weeks & 6 weeks respectively

### 3.2.2 Multiplication of shoots from shoot tip explants obtained from primary established shoot cultures

#### 3.2.2.1 Effect of different concentrations of BA and Kn singly or combinedly

In this experiment effect of different concentrations of BA or Kn singly or combinedly on shoot proliferation from shoot tip explants of primary established shoot culltures of *A. calamus* was studied. Each treatment consisted of three replications and in each replication 10-15 explants were used. Data on percentage of explants showing shoot proliferation, number of shoots per culture and average length of the longest shoot were recorded after 6 weeks of culture initiation and the results obtained are shown in Table 10.

From the results it was found that in all the treatments the explants formed shoots. When BA singly was used the percentage of explants responded ranged from 53.33 to 61.66%, the number of shoots per culture ranged from 3.44 to 4.39 and the average length of the longest shoot ranged from 2.29 to 3.88 cm. In case of Kn, the percentage of explants responded ranged from 37.33 to 45.32%, the number of shoots per culture ranged from 3.37 to 4.32 and the average length of the longest shoot ranged from 2.40 to 2.90 cm. In case of BA+Kn, the percentage of explants responded ranged from 41.66 to 66.66%, the number of shoots per culture ranged from 2.87 to 4.03 and the average length of the longest shoot ranged from 1.82 to 2.09 cm. Among nine treatments the highest percentage of response 61.66% was recorded in 1.0 mg/l BA+1.0 mg/l Kn. The highest number of shoots per culture was recorded in 3.0 mg/l BA and maximum length of longest shoot was recorded in 2.0 mg/l BA.

From the above results it might be concluded that MS media with 1.0 mg/l of BA+1.0 mg/l Kn was found to be the best for shoot proliferation from shoot tip explants of *A. calamus* in aspect of frequency of response and number of shoots but length of shoots was found highest in 2.0 mg/l BA.

**Table 10. Effect of different concentrations of BA with Kn singly or combinedly on shoot proliferation from shoot tip explants. Each treatment consisted of 3 replications and in each replication 10-15 explants were used. Data were recorded after 6 weeks of culture.**

| Growth regulators (mg/l) | % of explants showing proliferation | No. of shoots per culture | Average length of the longest shoot (cm) |
|---|---|---|---|
| BA | | | |
| 1.0 | 53.33 | 3.44 | 3.00 |
| 2.0 | 61.66 | 3.81 | 3.88 |
| 3.0 | 58.33 | 4.39 | 2.29 |
| Kn | | | |
| 1.0 | 37.33 | 3.37 | 2.40 |
| 2.0 | 45.32 | 3.86 | 2.90 |
| 3.0 | 44.59 | 4.32 | 2.52 |
| BA+Kn | | | |
| 1.0+1.0 | 66.66 | 4.03 | 2.01 |
| 2.0+1.0 | 50.00 | 3.30 | 2.09 |
| 2.0+2.0 | 41.66 | 2.87 | 1.82 |
| **LSD at 5% level** | **4.00** | **0.83** | **0.49** |

### 3.2.2.2 Effect of different concentration of BA with NAA or IAA

In this experiment effect of different concentrations of cytokinin BA with auxin NAA or IAA on shoot proliferation from *in vitro* grown shoot tip explants of *A. calamus* was studied. A total of eighteen different treatments were tested. Each treatment consisted of three replications and in each replication 12-15 explants were used. Data on percentage of explants showing shoot proliferation, number of shoots per culture and average length of the longest shoot were recorded after 5 weeks of culture initiation and the results obtained are shown in Table 11 and **Plate 9 (Figs. A and B).**

From the results it was found that in all the treatments the explants formed shoots but the frequency of response, number of shoot per culture and the length of shoot varied considerably. When BA and NAA were used combinedly the percentage of explants responded ranged from 60.11 to 98.90%, the number of shoots per culture ranged from 2.53 to 10.20 and the average length of the longest shoot ranged from 1.90 to 3.50 cm. In case of BA+IAA combination, the percentage of explants responded ranged from 33.33 to 88.59%, the number of shoots per culture ranged from 1.36 to 9.39 and the average length of the longest shoot ranged from 1.85 to 3.18 cm. Among the eighteen treatments the highest percentage of response 98.90% was recorded in 2.0 mg/l BA+0.1 mg/l NAA followed by 95.18% in 2.0 mg/l BA+0.5 mg/l NAA. But the number of shoots per culture was recorded highest in 2.0 mg/l BA + 0.5 mg/l NAA.

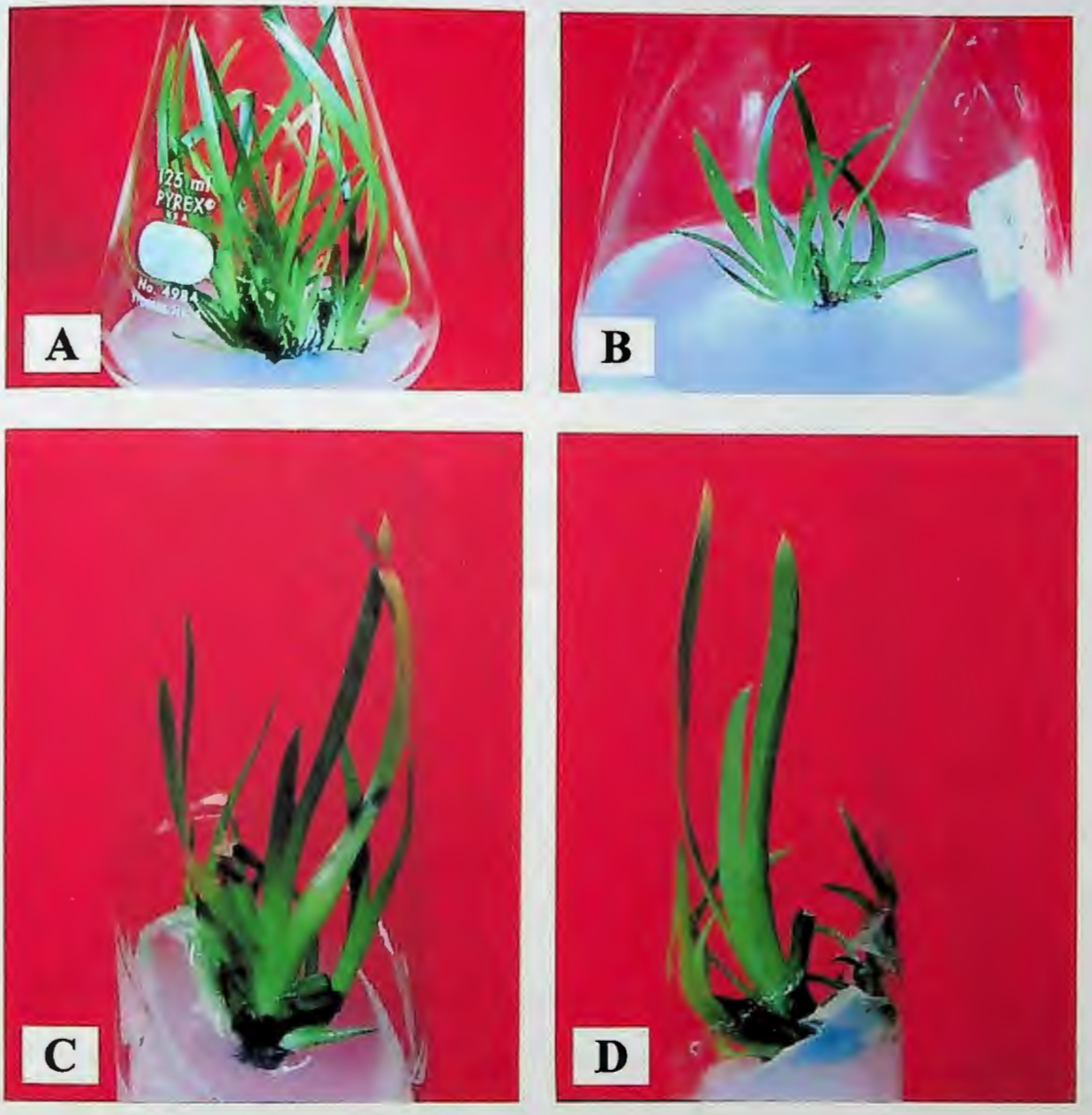
On average, between two combinations BA with NAA was found superior than BA with IAA in respect of all the three characters tested.

So, from the above results it might be concluded that the combination of 2.0 mg/l BA+0.1 mg/l NAA and also 2.0 mg/l BA + 0.5 mg/l NAA were found to be the best combination for shoot proliferation from *in vitro* shoot tip explants of *A. calamus*.

**Table 11. Effect of different concentration and combination of BA with NAA or IAA on shoot proliferation from shoot tip explants. Each treatment consisted of 3 replications and in each replication 12-15 explants were used. Data were recorded after 5 weeks of culture.**

| Growth regulators (mg/l) | % of explants showing proliferation | No. shoots per culture | Average length of the longest shoot (cm) |
|---|---|---|---|
| BA + NAA | | | |
| 1.0+0.1 | 60.90 | 4.12 | 2.37 |
| 1.0+0.5 | 73.08 | 2.53 | 2.91 |
| 1.0+1.0 | 60.11 | 4.66 | 3.25 |
| 2.0+0.1 | 98.90 | 9.23 | 2.05 |
| 2.0+0.5 | 95.18 | 10.20 | 2.12 |
| 2.0+1.0 | 90.33 | 6.87 | 3.50 |
| 3.0+0.1 | 86.56 | 7.99 | 2.65 |
| 3.0+0.5 | 80.55 | 5.75 | 2.23 |
| 3.0+1.0 | 66.39 | 4.36 | 1.90 |
| **Mean** | **77.11a** | **6.19a** | **2.55a** |
| BA+IAA | | | |
| 1.0+0.1 | 40.99 | 3.77 | 2.14 |
| 1.0+0.5 | 53.33 | 3.13 | 2.81 |
| 1.0+1.0 | 33.33 | 1.36 | 1.49 |
| 2.0+0.1 | 88.59 | 9.39 | 2.14 |
| 2.0+0.5 | 83.33 | 8.38 | 2.50 |
| 2.0+1.0 | 86.66 | 6.11 | 3.18 |
| 3.0+0.1 | 80.13 | 7.10 | 2.48 |
| 3.0+0.5 | 66.66 | 5.35 | 2.01 |
| 3.0+1.0 | 53.33 | 3.87 | 1.85 |
| **Mean** | **57.39b** | **5.38b** | **2.29a** |

# PLATE-9




Effect of auxin and cytokinin on shoot proliferation from *Acorus calamus* shoot tip explants.

**Fig. A:** MS medium containing 2.0 mg/l BA + 0.1 mg/l NAA.

**Fig.** B: MS medium containing 2.0 mg/l BA + 0.5 mg/l IAA.

**Fig. C:** MS medium containing 2.0 mg/l Kn + 0.05 mg/l NAA.

**Fig. D:** MS medium containing 2.0 mg/l Kn + 0.05 mg/l IAA.

### 3.2.2.3 Effect of different concentrations of Kn in combination with NAA or IAA

In this experiment effect of another cytokinin Kn with either NAA or IAA on shoot proliferation from *in vitro* grown shoot tip explants of *A. calamus* was studied. In this experiment nine different concentrations of Kn and NAA or IAA were tested. Each treatment consisted of three replications and in each replication 10-15 explants were used. Data on percentage of explants showing proliferation, number of shoots per culture and average length of the longest shoot were recorded after 6 weeks of culture initiation and the results obtained are shown in Table 12 and **Plate 9 (Figs. C and D).**

From the results it was found that in all the treatments the explants formed shoots. When Kn with NAA were used the percentage of explants responded ranged from 40.10 to 98.99%, the number of shoots per culture ranged from 1.10 to 8.34 and the average length of the longest shoot ranged from 1.35 to 3.50 cm. In case of Kn+IAA, the percentage of explants responded ranges from 33.13 to 97.33%, the number of shoots per culture ranged from 1.18 to 6.99 and the average length of the longest shoot ranged from 1.29 to 12.21 cm. Among eighteen treatments the highest percentage of response 98.99% was recorded with 2.0 mg/l Kn + 0.05 mg/l NAA followed by 97.33% with same concentration of Kn+IBA. Number of shoots per culture was also recorded high in the same combination.

On average, of the two combinations, Kn with NAA was found better than Kn with IAA. But no significantion was observed in number and length of shoots.

So, from the above results it might be concluded that the combination of 2.0 mg/l Kn + 0.05 mg/l NAA was found to be the best combination for shoot proliferation from *in vitro* grown shoot tip explants of *A. calamus*.

**Table 12. Effect of different concentration and combination of Kn with NAA or IAA on shoot proliferation from *in vitro* grown shoot tip explants. Each treatment consisted of 3 replications and in each replication 10-15 explants were used. Data were recorded after 6 weeks of culture.**

| Growth regulators (mg/l) | % of explants showing proliferation | No. shoots per culture | Average length of the longest shoot (cm) |
|---|---|---|---|
| KN+NAA | | | |
| 1.0+0.05 | 46.66 | 3.11 | 2.03 |
| 1.0+0.5 | 40.10 | 2.81 | 2.66 |
| 1.0+1.0 | 53.33 | 1.10 | 1.35 |
| 2.0+0.05 | 98.99 | 8.34 | 1.44 |
| 2.0+0.5 | 86.66 | 7.35 | 2.81 |
| 2.0+1.0 | 80.00 | 5.15 | 3.50 |
| 3.0+0.05 | 73.33 | 6.19 | 2.23 |
| 3.0+0.5 | 60.01 | 4.58 | 1.97 |
| 3.0+1.0 | 53.63 | 3.16 | 1.78 |
| **Mean** | **65.86a** | **4.64a** | **2.20a** |
| Kn + IAA | | | |
| 1.0+0.05 | 40.60 | 2.71 | 1.75 |
| 1.0+0.5 | 33.13 | 2.45 | 2.43 |
| 1.0+1.0 | 26.76 | 1.18 | 1.29 |
| 2.0+0.05 | 97.33 | 7.53 | 12.21 |
| 2.0+0.5 | 73.36 | 6.99 | 2.69 |
| 2.0+1.0 | 80.53 | 4.75 | 2.48 |
| 3.0+0.05 | 66.61 | 5.62 | 2.02 |
| 3.0+0.5 | 53.34 | 4.10 | 1.81 |
| 3.0+1.0 | 46.61 | 2.64 | 1.55 |
| **Mean** | **57.59b** | **4.22a** | **1.91a** |

Comparison between means of different characters was made using Duncan's multiple range test; the values in each column carrying different letters are significantly different at 5% level of probability.

### 3.2.2.4 Effect of different types and levels of sucrose

In this experiment effect of different types (Merck Ltd., Mumbai, India; *Phyto* Technology Laboratories, USA; BDH Chemicals Ltd., England and local market sugar) and concentration (10, 20, 30, 40, 50 and 60 g/l) of sucrose on shoot proliferation from shoot tip explants of *in vitro* proliferated shoots of *A. calamus* on MS medium containing BA 2.0 mg/l + NAA 0.5 mg/l was studied. Each treatment consisted of three replications and in each replication 10-12 explants were used. Data on percentage of explants showing shoot proliferation, number of shoots per culture and average length of the longest shoot were recorded after 6 weeks of culture initiation and the results obtained are shown in Table 13 and **Plate 10 (Fig. A)**.

From the results it was noted that in all the treatments the explants formed shoots but different parameters differed according to the type and level of sucrose. The percentage of explants responded ranged from 46.67 to 97.93%, 40.53 to 93.33%, 33.33 to 86.67% and 33.34 to 93.34% of Merck Ltd. (India), *Phyto* Technolgy (USA), BDH (UK) and local market sugar respectively. The number of shoots per culture ranged from 4.24 to 12.53, 3.85 to 9.05, 2.85 to 8.12 and 2.58 to 7.95 in Merck, *Phyto* Technolgy, BDH and local market respectively.

In each type of sucrose six levels were used. The highest percentage of explants showing proliferation were noticed 97.93% in Merck Ltd., Mumbai; 93.33% in *Phyto* Technology Laboratories, USA, 86.67% in BDH Chemicals Ltd., England and 93.34% in local market sugar. In all cases the highest results were observed at 40 g/l of sucrose.

On average, among the four brands Merck Ltd., *Phyto* Technology Laboratories gave the highest percentage of explants showing proliferation and it was 73.33% and other three brands showed equal response was observed.

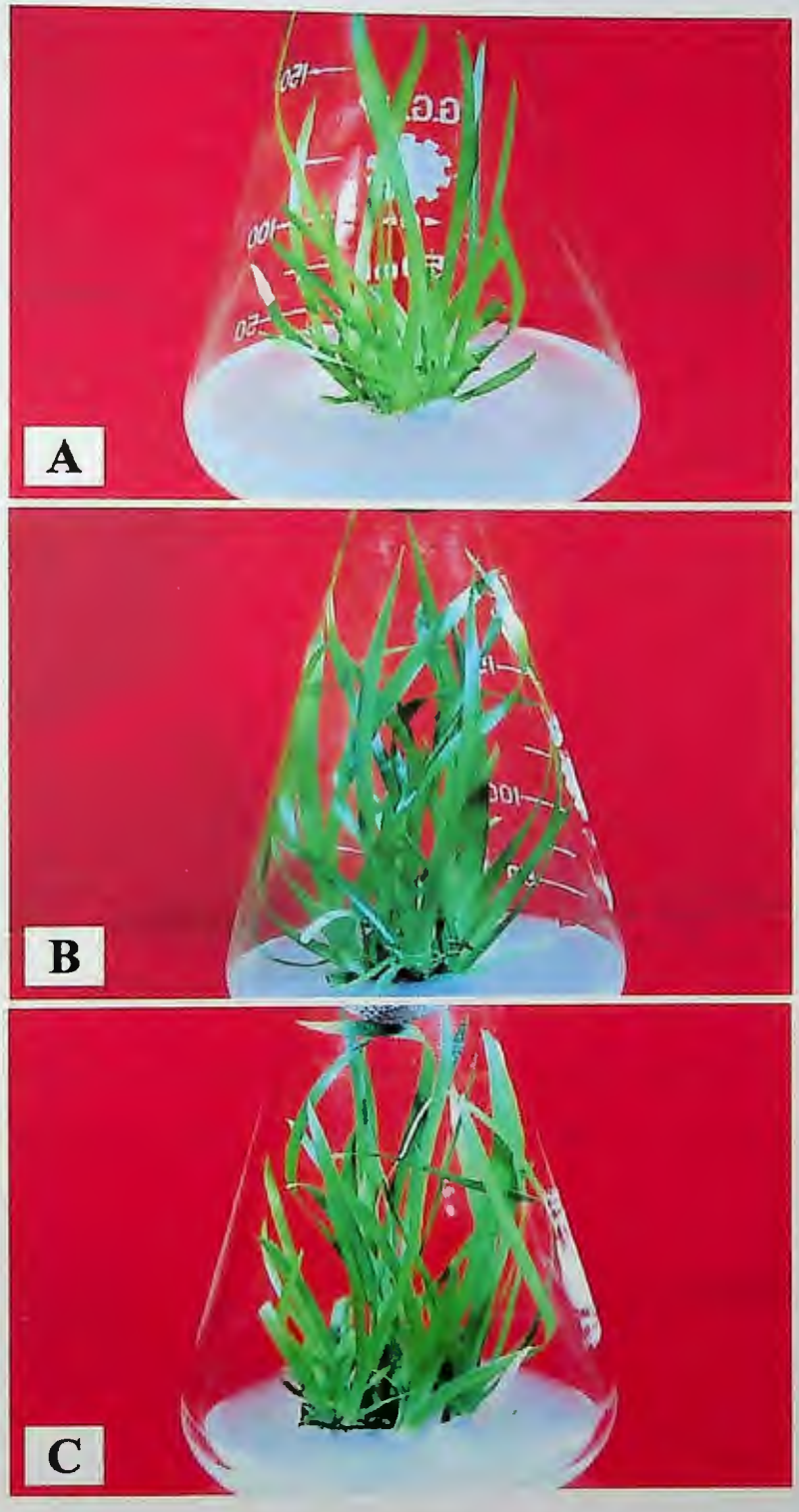
From the above results it might be concluded that 40 g/l sucrose of Merck Ltd., Mumbai showed the best results for shoot proliferation from shoot tip explants of *in vitro* proliferated shoots of *A. calamus* with BA 2.0 mg/l + NAA 0.5 mg/l.

**Table 13. Effect of different brands and concentration of sucrose on proliferation and growth of axillary shoots from shoot tip explants of *in vitro* proliferated shoots of *A. calamus* on MS medium containing BA 2.0 mg/l + NAA 0.5 mg/l. Each treatment consisted of 3 replications and in each replication 10-12 explants were used. Data were recorded after 6 weeks of culture.**

| Different brands of sucrose | Sucrose concentration g/l | % of explants showing proliferation | No. of shoots per culture | Average length of the longest shoot (cm) |
|---|---|---|---|---|
| | 10 | 46.67 | 4.24 | 2.05 |
| | 20 | 66.67 | 5.80 | 3.53 |
| Mark Ltd. | 30 | 93.33 | 8.20 | 4.91 |
| Mumbai | 40 | 97.93 | 12.53 | 5.20 |
| | 50 | 80.00 | 6.10 | 4.69 |
| | 60 | 53.33 | 5.39 | 2.95 |
| Mean | | **73.33a** | **7.04a** | **3.39a** |
| | 10 | 40.53 | 3.85 | 2.04 |
| *Phyto* | 20 | 60.10 | 5.44 | 3.25 |
| Technology | 30 | 46.68 | 9.05 | 2.45 |
| Laboratories™, | 40 | 93.33 | 9.20 | 2.85 |
| USA | 50 | 73.33 | 6.81 | 3.89 |
| | 60 | 46.67 | 5.39 | 2.85 |
| **Mean** | | **60.12b** | **6.62a** | **2.89ab** |
| | 10 | 33.33 | 2.85 | 1.89 |
| | 20 | 46.67 | 3.94 | 2.85 |
| BDH Chemical | 30 | 80.00 | 6.99 | 3.01 |
| Ltd. England | 40 | 86.67 | 8.12 | 2.25 |
| | 50 | 66.67 | 5.25 | 3.21 |
| | 60 | 40.00 | 3.10 | 2.21 |
| **Mean** | | **58.89b** | **5.04bc** | **2.57b** |
| Local market | 10 | 33.34 | 2.80 | 2.05 |
| sugar | 20 | 53.33 | 3.10 | 2.55 |
| | 30 | 80.00 | 5.50 | 2.50 |
| | 40 | 93.34 | 7.95 | 2.12 |
| | 50 | 60.00 | 4.65 | 4.01 |
| | 60 | 33.37 | 2.58 | 2.55 |
| **Mean** | | **58.95b** | **4.43c** | **2.63b** |

Comparison between means of different characters was made using Duncan's multiple range test; the values in each column carrying different letters are significantly different at 5% level of probability.

# PLATE-10

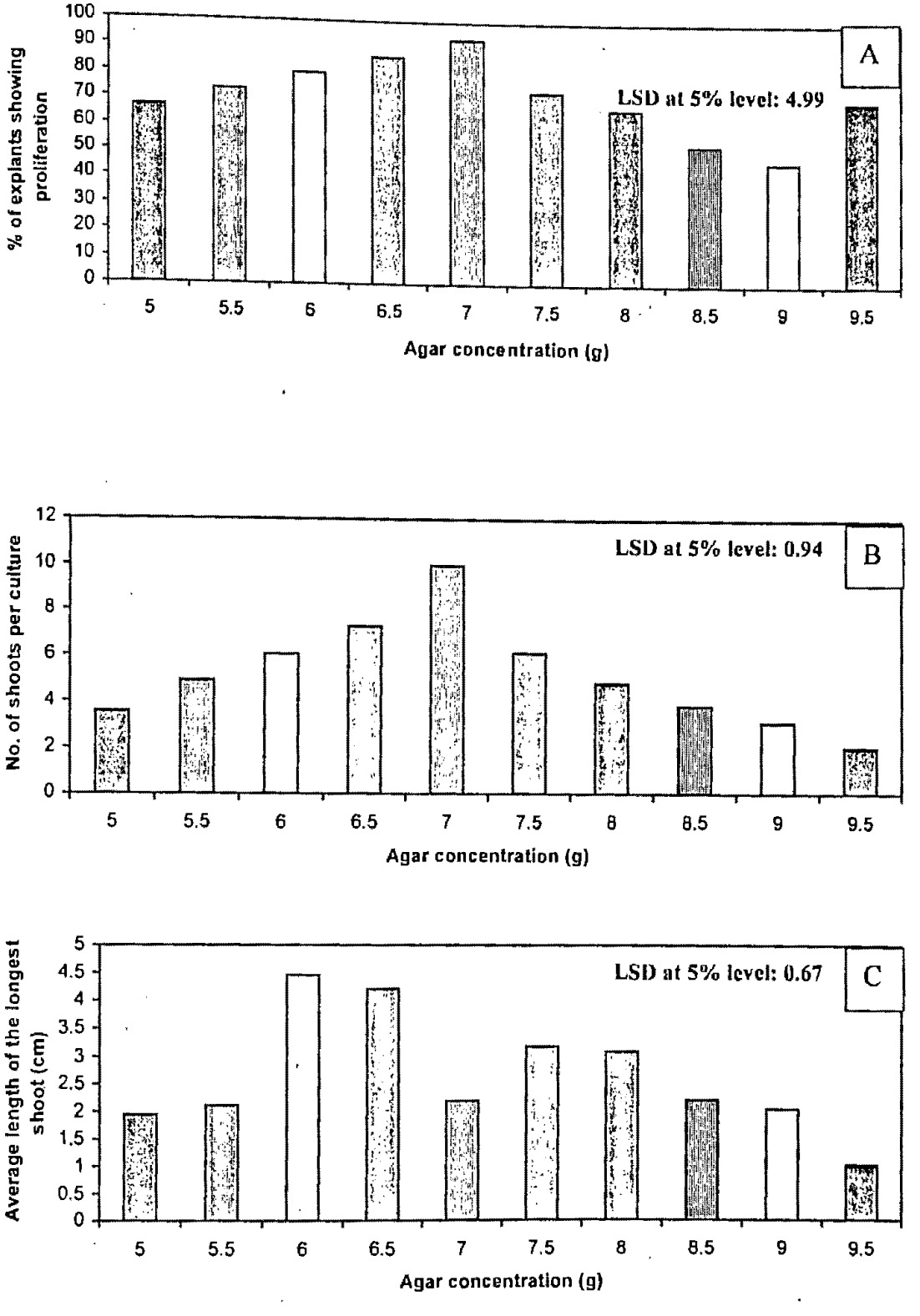



Mass shoot proliferation from *in vitro* grown shoot tip on MS + 2.0 mg/l BA + 0.05 mg/l NAA with 40 g/l sucrose after 6 weeks (**Fig. A**); on MS + 2.0 mg/l BA + 0.05 mg/l NAA with 7.0 g/l agar after 4 weeks (**Fig. B**); on MS + 2.0 mg/l BA + 0.05 mg/l NAA adjusted 5.5 level of pH after 4 weeks (**Fig. C**) of growth.

### 3.2.2.5 Effect of gelling agent at different concentration on shoot proliferation

The concentration and brand of gelling agent also affect the growth and development of *in vitro* culture. Therefore, it is necessary to standardize the gelling agent's strength for the maximum production of shoots. In this investigation agar was used as gelling agent and the nodal explants were collected from *in vitro* grown cultures. These explants were cultured on MS medium at ten different strengths of agar (E. Merck, India) viz. 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0 and 9.5 g/l and data were collected after six weeks of culture from different agar strengths.

The highest percentage of explants showing shoot proliferation of shoots and highest number of shoots per culture were 95% and 9.8 recorded in the medium containing 7.0 g/l agar. The medium containing 9.0 g/l agar was found to produce the lowest percentage of shoot proliferation. The highest average length of longest shoot per culture was 4.3 also recorded at 6.0 g/l agar. It was also noticed that in all the treatments, levels of agar concentration from 8.0-9.5 g/l the medium became too hard which was not suitable for proper growth and development of *in vitro* plantlets. According to the above observation and records it was clear that agar concentration of 7.0 g/l showed the best results for *in vitro* shoot proliferation in *A. calamus* (**Graph 2** and **Plate 10, Fig. B**).

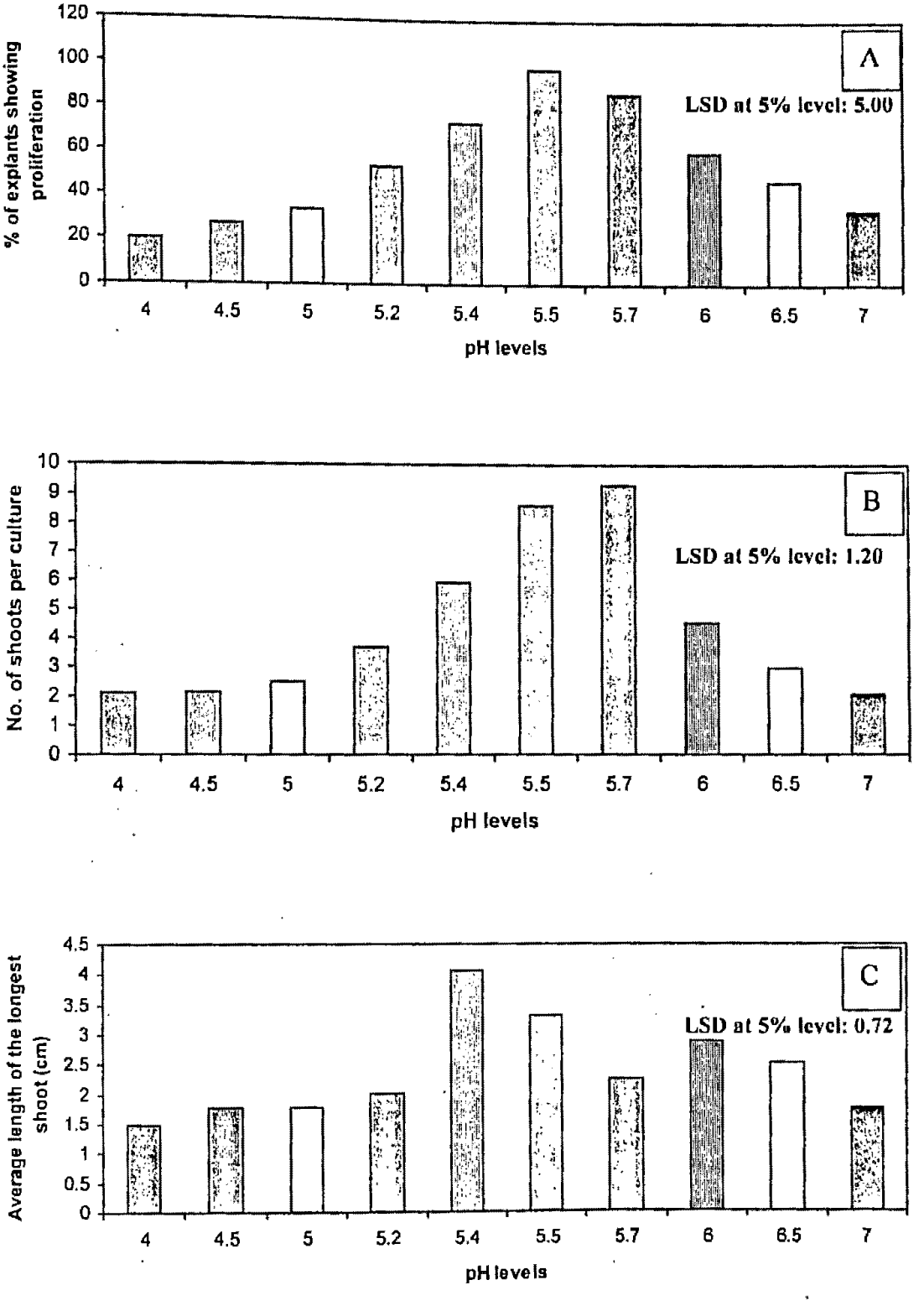



**Graph 2.** Effect of different strengths of agar on shoot proliferation in *A. calamus*. A. Percentage of explants showing shoot proliferation, B. Number of shoots per culture C. Average length of the longest shoot (cm).

### 3.2.2.6 Effect of different levels of pH in the medium on shoot proliferation

In the *in vitro* proliferation system, pH of the medium is an important factor. To optimize the pH level, medium was prepared with different values of pH and explants were collected from the *in vitro* grown shoots. The explants were cultured on MS medium having ten different pH levels viz. 4.0, 4.5, 5.0, 5.2, 5.4, 5.5, 5.7, 6.0, 6.5 7.0. At pH 4.0, 4.5 and 5.0 filter paper bridges were used, as the media do not form gel. Media with these levels of pH were used to standardize the optimum pH value for MS medium for *Acorus calamus* shoot culture. Percentage of proliferated shoots, number of shoots per culture and average length of longest shoot were recorded after 6 weeks of culture initiation. The highest percentage of explants formed shoots was recorded 100% in medium having pH value 5.5 (**Plate 10, Fig C**). But the highest number (9.0) of shoots per culture was recorded in pH 5.7. The lowest percentage of explants showing shoot proliferation was 20 recorded at pH value 4.0. But the lowest numbers of shoots per culture (an average of 2) was counted at the pH levels of 4.0 and 7.0 (**Graph 3**).

It is evident from the results that the pH level not only affected the frequency of culture showing proliferation, but also the number and growth of proliferated shoots were also affected. It also, reveals that the shoots of *Acorus calamus* preferred slightly acidic pH medium.




**Graph 3.** Effect of pH levels of culture media on shoot proliferation in *A. calamus* A. Frequency of explants showing proliferation, B. No. of shoots per culture C. Average length of the longest shoot (cm).

### 3.2.3 Rooting of *in vitro* proliferated shoots

After multiplication of shoot culture rooting was induced to raise fullfledged plantlets. In this experiment effect of different concentration and combination of NAA and IBA singly or combinedly on *in vitro* root formation from microcuttings in *A. calamus* was studied. Each treatment consisted of three replications and in each replication 10-12 explants were used. Data on percentage of explants showing proliferation, number of shoots per culture and average length of the longest shoot were recorded after 5 weeks of culture initiation and the results obtained are shown in Table 14 and **Plate 11 (Figs. A, B** and **C)**.

From the results it was found that in all the treatments the explants formed shoots except in two treatments, 0.1 mg/l NAA and 0.1 mg/l IBA. When NAA singly was used the percentage of explants responded ranged from 0 to 25.53, the number of root per microshoot ranged from 0 to 2.26 and the average length of the longest root ranged from 0 to 2.15 cm. In case of IBA singly was used the percentage of explants responded ranged from 0 to 40.51%, the number of root per micro-shoots ranged from 0 to 3.15 and the average length of the longest root ranged from 0 to 2.28 cm. When NAA with IBA was used combinedly the rooting performance was not improved. In all cases of NAA and IBA concentrations slight to profuse callusing was found at the base of the cultured shoot and profuse callusing was recorded in case of NAA. Among the nine treatments the highest percentage of response 40.51% was recorded in 1.0 mg/l IBA followed by 35.81% with 0.5 mg/l IBA. Number of roots per cutting and length of the longest root were also recorded highest in the same concentration. Delayed rooting (10-13 days) was observed in NAA supplemented media but days to rooting was reduced when IBA was used.

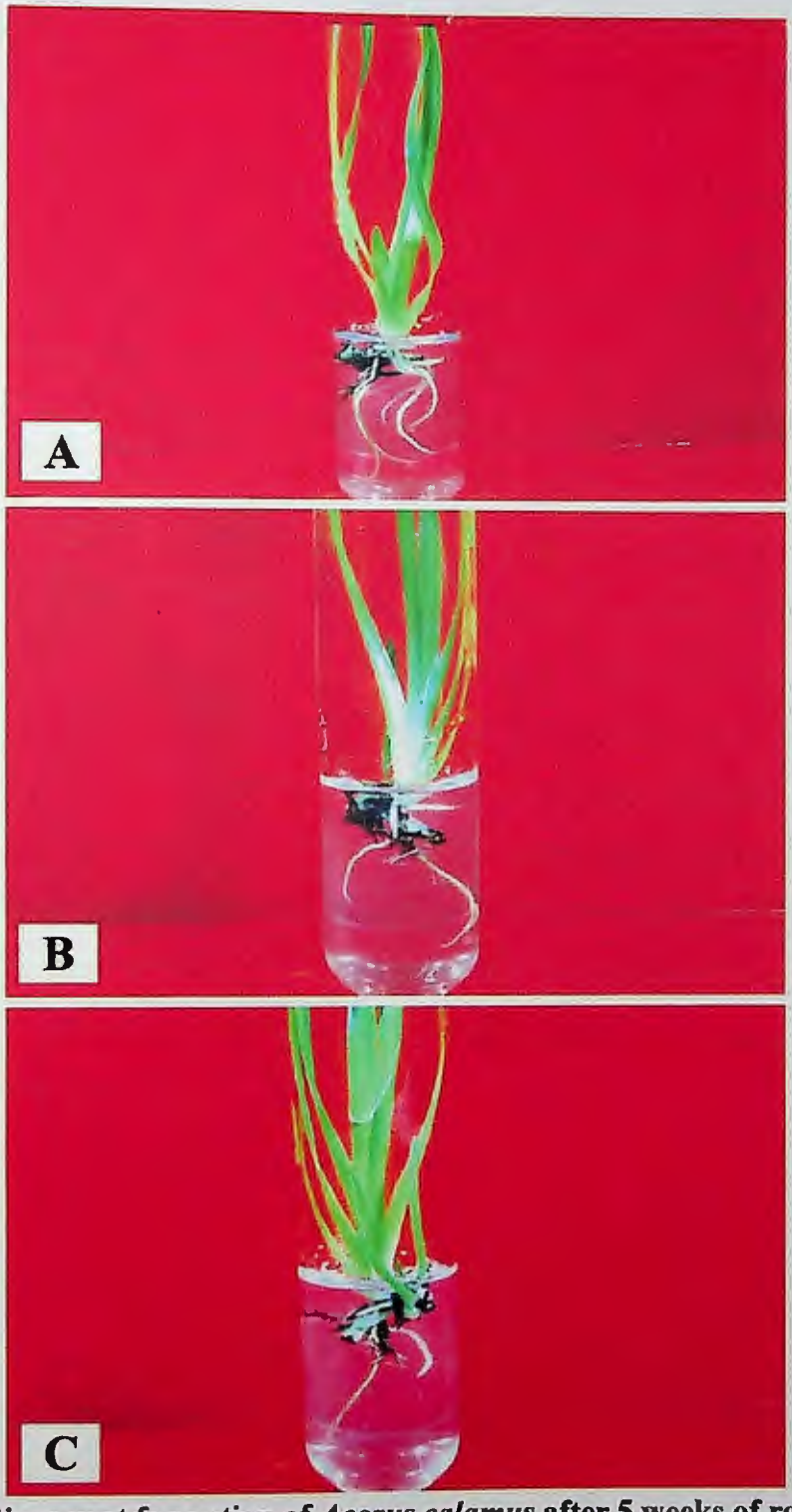
From the above results it might be concluded that medium with IBA at 1.0 mg/l was found to be the best media formulation for adventitious root formation from microcuttings in *A. calamus*.

**Table 14. Effect of different concentration and combination of NAA and IBA on *in vitro* root formation from microcuttings of *A. calamus*. Each treatment consisted of 3 replications and in each replication 10-12 explants were used. Data were recorded after 5 weeks of culture.**

| Growth regulators (mg/l) | % of root formation | No. of roots per cutting | Average length of the longest root (cm) | Days to root formation | Callus formation of cutting base |
|---|---|---|---|---|---|
| NAA | | | | | |
| 0.1 | - | - | - | - | +++ |
| 0.5 | 25.53 | 2.26 | 2.15 | 10-13 | +++ |
| 1.0 | 20.33 | 2.12 | 2.15 | 10-12 | +++ |
| IBA | | | | | |
| 0.1 | - | - | - | - | + |
| 0.5 | 35.81 | 3.11 | 2.28 | 7-10 | + |
| 1.0 | 40.51 | 3.15 | 2.25 | 7-10 | ++ |
| NAA+IBA | | | | | |
| 0.5 | 30.39 | 2.35 | 2.15 | 7-10 | + |
| 1.0+0.5 | 30.69 | 2.15 | 2.00 | 7-10 | - |
| 1.0+1.0 | 25.59 | 2.36 | 2.12 | 7-10 | - |

+, ++, +++, indicate slight, considerable, profuse callusing, respectively, -indicates no response.

# PLATE-11



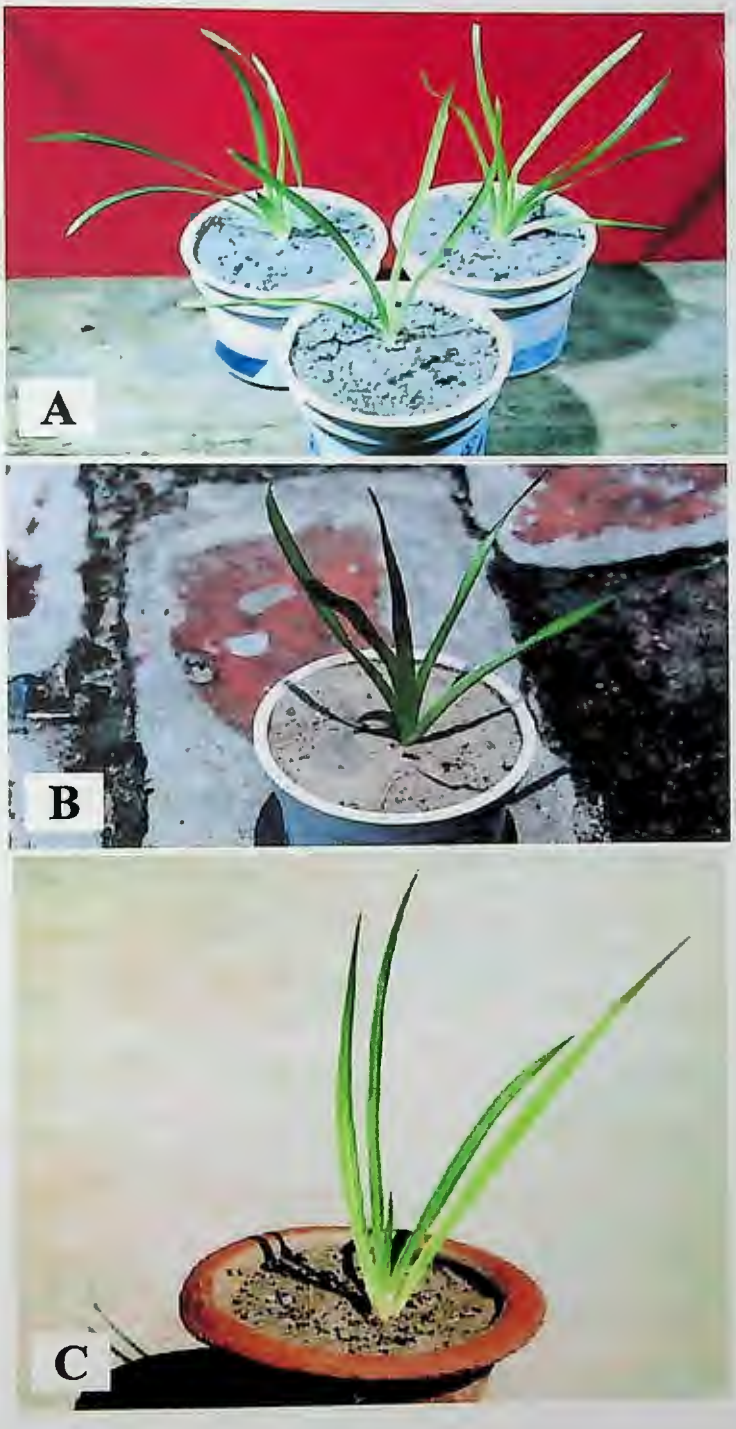

**Adventitious root formation of *Acorus calamus* after 5 weeks of rooting.**

**Fig. A:** MS medium with 1.0 gm/l IBA.
**Fig. B**: MS medium with 1.0 mg/l NAA+0.5 mg/l IBA.
**Fig. C**: MS medium with 0.5 mg/l NAA.

### 3.2.4 Acclimatization and establishment of plantlets on soil

When the root system of the *in vitro* regenerated plantlets developed sufficiently, they were transferred to soil. After bringing out from the culture vessels, root system of regenerated plantlets were made agar-gel free by continuous flushing of running tap water. The plantlets were then made ready for transplantation after cleaning of dead and decolourized tissues and parts. The plantlets were initially planted in especially made perforated small plastic pots, containing coco-peat or sun-sterilized sand, soil and humus in 1:2:1 ratios. The potted plants were then watered adequately and covered with perforated polythene bag and kept in the laboratory condition for 10-15 days (**Plate 12, Fig. A**). By this time the transplants adapted themselves to the *ex vitro* environment. After few days of indoor acclimatization the plantlets from the perforated small plastic pots were transplanted to comparatively larger pots and kept in semi-outdoor condition (**Plate 12, Fig. B**). After achieving sufficient growth the plantlets they were then transferred to the field soil. Among the regenerates transplanted, about 68% of the transferred plants survived and acclimatized themselves successfully on the soil under *ex vitro* condition (**Plate 12, Fig. C**). The rest of the transplants could not survive under the *ex vitro* condition, mainly due to transplantation shock and environmental stress under outdoor condition.

# PLATE-12




**Growth of transplants of *Acorus calamus*.**

**Fig. A:** After 2 weeks indoor condition.

**Fig. B:** After 4 weeks semi-outdoor condition.

**Fig. C:** After 5 weeks under the *ex vitro* condition.

## 3.3. *S. rebaudiana* Bertoni.

### 3.3.1 Primary establishment of shoot culture from nodal segments of pot grown plants

For primary establishment of shoot cultures, nodal segments from pot growing plants of *S. rebaudiana* Bertoni. Were used (**Plate 13, Figs. A, B and C**). Before clture the explants were surface sterilized in 0.1% $HgCl_2$ solution and duration of treatment was fixed for ten minutes. In this treatment of $HgCl_2$ 75-80% of cultured explants was found to survive and produced shoots. The surface sterilized nodal segments were cultured to induce axillary shoots. In this experiment effect of different concentrations of BA with Kn singly or combinedly on shoot proliferation from shoot tip explants was studied. A total of fifteen treatments were used in this experiment. Each treatment consisted of three replications and in each replication 10-15 explants were used. Data on percentage of explants showing shoot proliferation, number of shoots per culture and average length of the longest shoot were recorded after 6 weeks of culture initiation and the results obtained are shown in Table 15.

From the results it was found that in all the treatments the explants formed shoots. While BA singly was used the percentage of explants responded ranged from 37.33 to 60.00%, the number of shoots per culture ranged from 3.20 to 4.45 and the average length of the longest shoot ranged from 3.00 to 3.90 cm. In case of Kn, the percentage of explants responded ranged from 33.33 to 46.66%, the number of shoots per culture ranged from 3.37 to 4.40 and the average length of the longest shoot ranged from 2.14 to 3.80 cm. In case of BA+Kn, the percentage of explants responded ranged from 33.33 to 73.33%, the number of shoots per culture ranged from 2.87 to 4.50 and the average length of the longest shoot ranged from 1.82 to 3.50 cm. Among the fifteen treatments the highest percentage of response 73.33% was

recorded with 1.5 mg/l BA+1.0 mg/l Kn. Number of shoot per culture and average length of longest shoot per culture were also recorded highest in the same concentration and combinations.

From the above results it might be concluded that MS medium with 1.5 mg/l of BA+1.0 mg/l Kn was found to be the best for shoot proliferation from grown shoot tip explants of *S. rebaudiana*. On average, of the two cytokinins BA showed better performance than Kn and when BA was used combined with Kn the percentage of response increased significantly from Kn but not from BA.

# PLATE-13

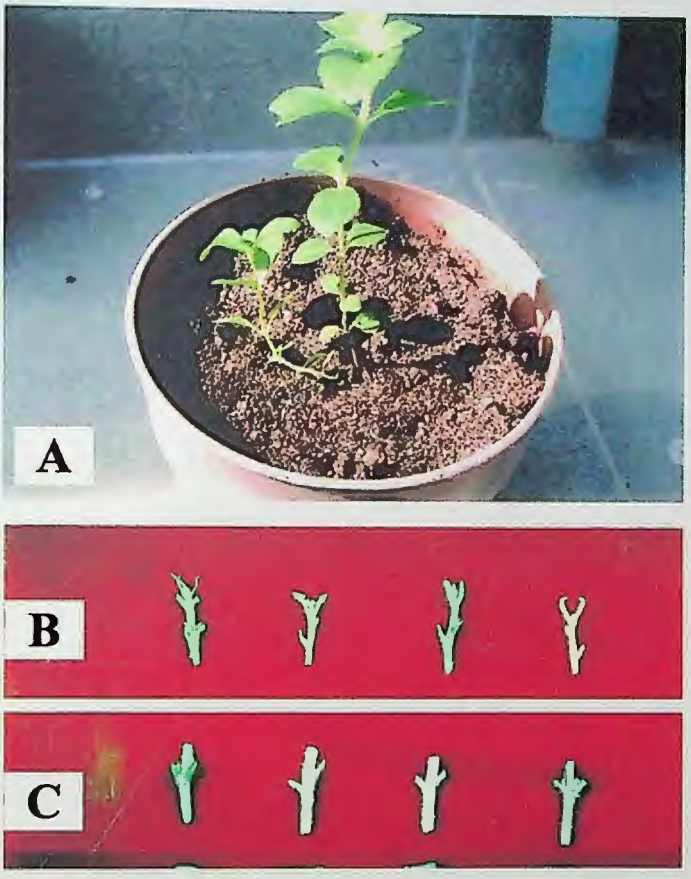



**Fig. A:** Morphological features of pot grown *Stevia rebaudiana* Bertoni. **Fig. B:** Shoot tip explants; **Fig. C:** Nodal segments

**Table 15. Effect of different concentrations of BA with Kn singly or combinedly on shoot proliferation from shoot tip explants. Each treatment consisted of 3 replications and in each replication 10-15 explants were used. Data were recorded after 6 weeks of culture.**

| Growth regulators (mg/l) | % of explants showing proliferation | No. of shoots per culture | Average length of the longest shoot (cm) |
|---|---|---|---|
| BA | | | |
| 1.0 | 40.00 | 3.20 | 3.00 |
| 1.5 | 53.33 | 3.44 | 3.71 |
| 2.0 | 60.00 | 4.45 | 3.90 |
| 2.5 | 46.66 | 3.81 | 3.05 |
| 3.0 | 37.33 | 3.14 | 3.29 |
| **Mean** | **47.46a** | **3.61a** | **3.31a** |
| Kn | | | |
| 1.0 | 40.00 | 4.03 | 2.14 |
| 1.5 | 46.66 | 3.37 | 2.40 |
| 2.0 | 44.59 | 4.40 | 3.80 |
| 2.5 | 40.00 | 3.86 | 3.30 |
| 3.0 | 33.33 | 3.10 | 2.52 |
| **Mean** | **40.09b** | **3.75a** | **2.83b** |
| BA+Kn | | | |
| 1.0+1.0 | 46.66 | 3.30 | 2.30 |
| 1.5+1.0 | 73.33 | 4.50 | 3.50 |
| 2.0+1.5 | 53.33 | 4.03 | 3.154 |
| 2.5+2.0 | 41.66 | 3.30 | 2.09 |
| 3.0+2.5 | 33.33 | 2.87 | 1.82 |
| **Mean** | **49.66a** | **3.60a** | **2.57b** |

### 3.3.2 Proliferation of axillary shoots from *in vitro* grown nodal segments

#### 3.3.2.1 Effect of different concentration of BA with NAA or IAA

Further multiplication of shoots was done using nodal segments of *in vitro* grown shoots obtained from primary established shoot culture. For standardization of optimum culture condition for shoot multiplication several experiments were conducted. This experiment was conducted to see the effect of different concentration and combinations of BA with NAA or IAA on axillary shoot proliferation from nodal explants from *in vitro* grown shoots of *S. rebaudiana*. In each combination twelve different hormonal treatments were tested. Each treatment consisted of three replications and in each replication 10-12 explants were used. Data on percentage of explants showing shoot proliferation, number of shoots per culture and average length of the longest shoot were recorded after 6 weeks of culture and the results obtained are shown in Table 16 and **Plate 14 (Figs. A, B** and **C)**.

From the results it was found that in all the treatments the explants formed shoots except in three treatments. When BA with NAA were used the percentage of explants responded ranged from 0 to 98.33%, the number of shoots per culture ranged from 0 to 9.10 and the average length of the longest shoot ranged from 0 to 3.95 cm but in case of BA+IAA, the percentage of explants responded ranged from 0 to 80.13, the number of shoots per culture ranged from 0 to 5.0 and the average length of the longest shoot ranged from 0 to 3.10 cm. The highest percentage of response 98.33% was recorded in MS with 1.0 mg/l BA+0.1 mg/l NAA followed by 85.13% with 1.0 mg/l BA+0.2 mg/l NAA. Number of shoots per culture was also recorded highest in the same combination. In all the treatments slight to profuse callus formation at the base of the cultured explants was observed. Generally profuse callusing inhibited frequency of shoot formation and number of shoot per explant.

On average, of the two combinations BA with NAA, and BA with IAA, the percentages of explants showing proliferation were 65.73% and 57.70% respectively. Average length of longest shoot was 2.88 and 2.42 cm respectively.

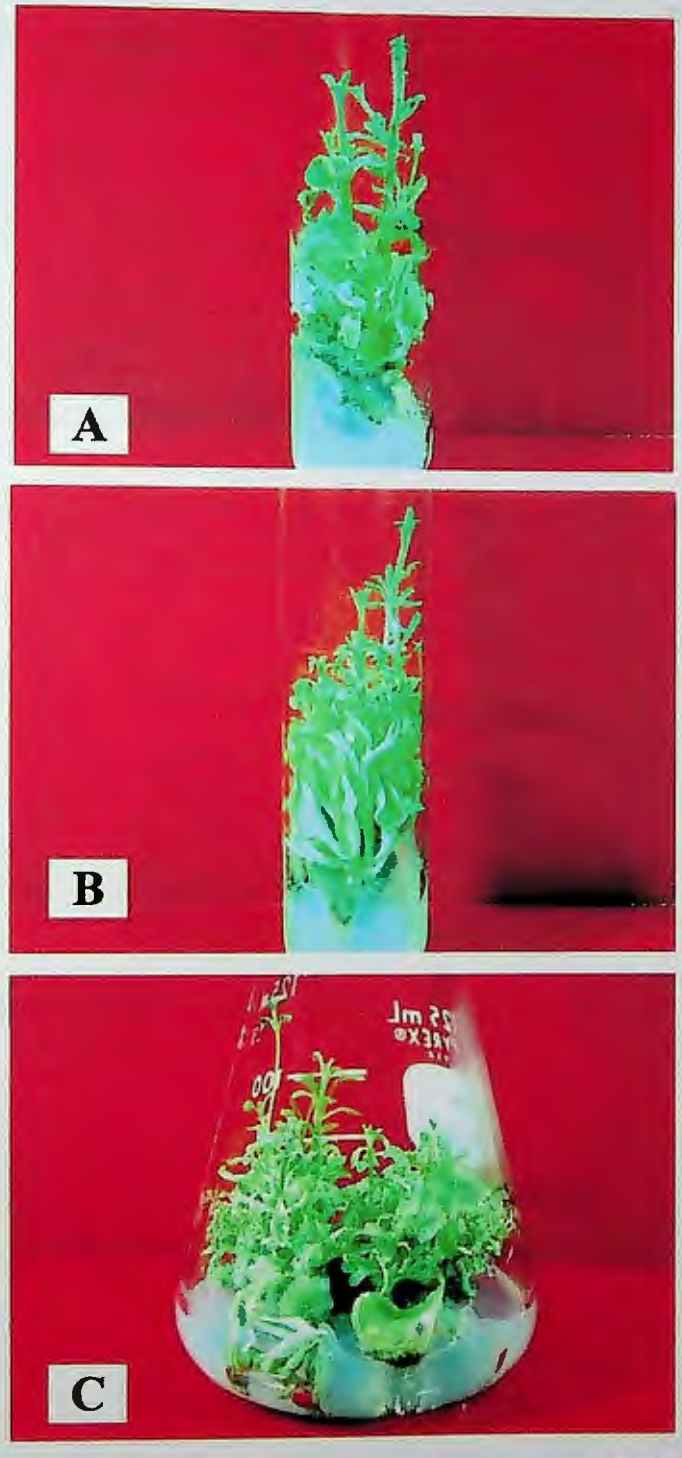
So, from the above results it might be concluded that the combination of 1.0 mg/l BA+0.1 mg/l NAA was found to be the best combination for shoot proliferation from *in vitro* grown nodal explants in *S. rebaudiana.*

**Table 16. Effect of different concentration and combination of BA with NAA or IAA on axillary shoot proliferation from *in vitro* grown nodal explants in *S. rebaudiana*. Each treatment consisted of 3 replications and in each replication 10-12 explants were used. Data were recorded after 6 weeks of culture.**

| Growth regulators (mg/l) | % of explants showing proliferation | No. of shoots per culture | Average length of the longest shoot (cm) | Callus formation |
|---|---|---|---|---|
| **BA+NAA** | | | | |
| 0.5+0.1 | 80.34 | 7.45 | 2.20 | + |
| 0.5+0.2 | 75.87 | 6.15 | 2.80 | ++ |
| 0.5+0.5 | 45.51 | 2.85 | 3.45 | +++ |
| 0.5+1.0 | - | - | - | +++ |
| 1.0+0.1 | 98.33 | 9.10 | 2.30 | + |
| 1.0+0.2 | 85.13 | 8.45 | 3.95 | ++ |
| 1.0+0.5 | 50.05 | 3.50 | 3.45 | +++ |
| 1.0+1.0 | - | - | - | +++ |
| 2.0+0.1 | 60.15 | 4.25 | 2.90 | + |
| 2.0+0.2 | 55.39 | 3.10 | 2.60 | ++ |
| 2.0+0.5 | 40.83 | 2.75 | 2.30 | +++ |
| 2.0+1.0 | - | - | - | +++ |
| **Mean** | **65.73** | **5.29** | **2.88** | |
| **BA+IAA** | | | | |
| 0.5+0.1 | 75.93 | 4.45 | 2.35 | + |
| 0.5+0.2 | 70.10 | 4.30 | 2.30 | ++ |
| 0.5+0.5 | 40.88 | 2.10 | 2.20 | +++ |
| 0.5+1.0 | - | - | - | +++ |
| 1.0+0.1 | 70.59 | 3.35 | 2.91 | + |
| 1.0+0.2 | 80.13 | 5.00 | 3.10 | + |
| 1.0+0.5 | 55.25 | 1.50 | 2.55 | +++ |
| 1.0+1.0 | - | - | - | +++ |
| 2.0+0.1 | 50.81 | 1.90 | 2.03 | + |
| 2.0+0.2 | 40.55 | 1.50 | 2.00 | ++ |
| 2.0+0.5 | 35.03 | 1.25 | 2.36 | +++ |
| 2.0+1.0 | - | - | - | +++ |
| **Mean** | **57.70** | **2.82** | **2.42** | |
| **Grand mean** | **61.72** | **4.06** | **2.65** | |
| **LSD at 5% level** | **4.89** | **0.39** | **0.71** | |

+, ++, +++, indicate slight, considerable, profuse callusing, respectively, -indicates no response.

# PLATE-14




Axillary shoot proliferation from the nodal explants of *S. rebaudiana* on MS+1.0 mg/l BA+0.1 mg/l NAA (**Figs. A, B** and **C**) after 2, 4 and 6 weeks respectively.

### 3.3.2.2 Effect of different concentration of Kn with NAA or IAA

In this experiment effect of different concentration and combination of Kn with NAA or IAA on auxillary shoot proliferation from *in vitro* grown nodal explants of *S. rebaudiana* was studied. In each combination of cytokinin and auxin twelve treatments were tested. Each treatment consisted of three replications and in each replication 12-15 explants were used. Data on percentage of explants showing proliferation, number of shoots per culture and average length of the longest shoot were recorded after 5 weeks of culture initiation and the results obtained are shown in Table 17.

From the results it was found that in all the treatments the explants formed shoots except in three treatments of each of the combination. When Kn with NAA were used the percentage of explants responded ranged from 0 to 65.93%, the number of shoots per culture ranged from 0 to 6.25 and the average length of the longest shoot ranged from 0 to 3.50 cm. In case of Kn + IAA, the percentage of explants responded ranged from 0 to 55.93%, the number of shoots per culture ranged from 0 to 3.90% and the average length of the longest shoot ranged from 0 to 3.64 cm. The highest percentage of response 65.93% was recorded with 1.0 mg/l Kn + 0.1 mg/l NAA followed by 55.93 with same concentration of Kn + IAA. Number of shoots per culture was also recorded highest in the same treatment of Kn + NAA.

Of the two combinations Kn with NAA showsd better results than Kn with IAA.

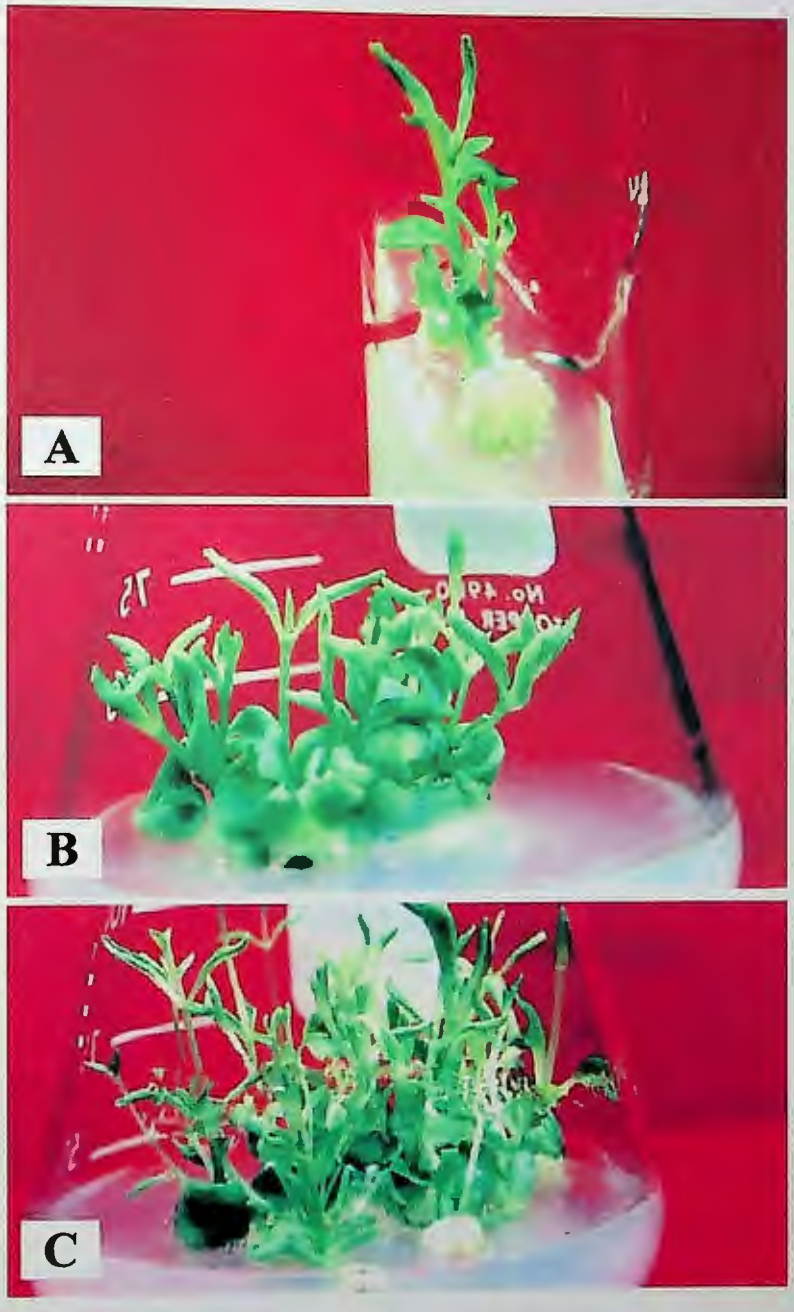
So, from the above results it might be concluded that the combination of 1.0 mg/l Kn + 0.1 mg/l NAA was found to be the best combination for shoot proliferation from *in vitro* grown nodal explants of *S. rebaudiana* **(Plate 15, Figs. A, B and C).**

**Table 17. Effect of different concentration and combination of Kn with NAA or IAA on axillary shoot proliferation from *in vitro* grown nodal explants in *S. rebaudiana*. Each treatment consisted of 3 replications and in each replication 12-15 explants were used. Data were recorded after 5 weeks of culture.**

| Growth regulators (mg/l) | % of explants showing proliferation | No. of shoots per culture | Average length of the longest shoot (cm) | Callus formation |
|---|---|---|---|---|
| **Kn+NAA** | | | | |
| 0.5+0.1 | 55.89 | 4.50 | 2.45 | + |
| 0.5+0.2 | 50.33 | 3.25 | 3.50 | + |
| 0.5+0.5 | 40.51 | 2.25 | 1.93 | +++ |
| 0.5+1.0 | - | - | - | +++ |
| 1.0+0.1 | 65.93 | 6.25 | 2.65 | + |
| 1.0+0.2 | 50.81 | 5.10 | 3.35 | + |
| 1.0+0.5 | 45.21 | 2.35 | 3.05 | +++ |
| 1.0+1.0 | - | - | - | +++ |
| 2.0+0.1 | 35.54 | 2.30 | 2.60 | + |
| 2.0+0.2 | 30.33 | 2.20 | 2.14 | + |
| 2.0+0.5 | 25.08 | 1.90 | 1.10 | +++ |
| 2.0+1.0 | - | - | - | ++ |
| **Mean** | **44.40** | **3.34** | **2.58** | |
| **Kn+IAA** | | | | |
| 0.5+0.1 | 50.19 | 2.45 | 2.25 | + |
| 0.5+0.2 | 35.00 | 2.25 | 2.90 | + |
| 0.5+0.5 | 30.42 | 2.00 | 1.15 | +++ |
| 0.5+1.0 | - | - | - | +++ |
| 1.0+0.1 | 55.93 | 3.90 | 3.64 | + |
| 1.0+0.2 | 45.87 | 2.75 | 2.91 | + |
| 1.0+0.5 | 35.21 | 2.20 | 2.13 | +++ |
| 1.0+1.0 | - | - | - | +++ |
| 2.0+0.1 | 35.11 | 1.25 | 3.10 | + |
| 2.0+0.2 | 25.39 | 2.15 | 2.15 | + |
| 2.0+0.5 | 20.08 | 1.80 | 1.08 | +++ |
| 2.0+1.0 | - | - | - | +++ |
| **Mean** | **37.02** | **2.31** | **2.37** | |
| **Grand mean** | **40.71** | **2.83** | **2.48** | |
| **LSD at 5% level** | **5.08** | **1.03** | **0.91** | |

+, ++, +++, indicate slight, considerable, profuse callusing, respectively, -indicates no response.

# PLATE-15




Axillary shoot proliferation from the nodal explants on MS + 1.0 mg/l Kn + 0.1 mg/l NAA after 2 weeks (**Fig. A**), after 4 weeks (**Fig. B**) and after 5 weeks (**Fig. C**) of growth.

### 3.3.3 Proliferation of adventatious shoots from internode explants of *in vitro* grown shoots

#### 3.3.3.1 Effect of BA alone or in combination with NAA , IAA or IBA

In this experiment effect of different concentration of BA singly or in combination with NAA, IAA or IBA on induction of adventitious shoots from *in vitro* grown internode explants in *S. rebaudiana* was studied. Three concentrations of BA and nine hormonal treatments of the three combinations were used in this experiment. Each treatment consisted of three replications and in each replication 10-12 explants were used. Data on percentage of explants showing shoot proliferation, number of shoots per culture and average length of the longest shoot were recorded after 5 weeks of culture and the results obtained are shown in Table 18 (**Plate 16, Figs. D, E and F**).

From the results it was found that BA alone failed to induce any adventatious shoots. Out of nine treatments five treatments of each of the three combinations also failed to induce any adventitious shoots. Slight to profuse callusing was observed before inducing adventitious shoots. Generally shoots developed after formation of callus on surface of the explants. While BA with NAA were used the percentage of explants responded ranged from 0 to 80.97%, the number of shoots per culture ranged from 0 to 12.60 and the average length of the longest shoot ranged from 0 to 5.20 cm. In case of BA+IAA, the percentage of explants responded ranges from 0 to 55.33%, the number of shoots per culture ranged from 0 to 6.35 and the average length of the longest shoot ranged from 0 to 3.25 cm. In case of BA with IBA the percentage of explants responded ranges from 0 to 50.31%, the number of shoots per culture ranged from 0 to 5.25 and the average length of the longest shoot ranged from 0 to 3.15 cm. Among the different treatments the highest percentage of response 98.33% was recorded in MS with 1.0 mg/l BA+0.1 mg/l

NAA followed by 80.97 with 1.0 mg/l BA+0.2 mg/l NAA. Number of shoots per culture was also recorded highest in the same combination

So, from the above results it might be concluded that out of three combinations BA+NAA showed netter results and the combination of 1.0 mg/l BA+0.1 mg/l NAA was found to be the best combination for induction of adventitious shoots from *in vitro* grown internode explants in *S. rebaudiana*.

**Table 18. Effect of different concentration of BA singly or in combination with NAA, IAA or IBA for induction of adventitious shoots from *in vitro* grown internode explants in *S. rebaudiana*. Each treatment consisted of 3 replications and in each replication 10-12 explants were used. Data were recorded after 5 weeks of culture.**

| Growth regulators (mg/l) | % of explants showing proliferation | No. of shoots per culture | Average length of the longest shoot (cm) | Callus formation |
|---|---|---|---|---|
| **BA** | | | | |
| 0.5 | - | - | - | - |
| 1.0 | - | - | - | - |
| 2.0 | - | - | - | - |
| **BA+NAA** | | | | |
| 0.5+0.1 | 50.03 | 8.20 | 5.20 | + |
| 0.5+0.2 | 40.80 | 7.40 | 4.10 | + |
| 0.5+0.5 | - | - | - | +++ |
| 1.0+0.1 | 75.50 | 10.45 | 3.10 | ++ |
| 1.0+0.2 | 80.97 | 12.60 | 2.20 | + |
| 1.0+0.5 | - | - | - | +++ |
| 2.0+0.1 | - | - | - | +++ |
| 2.0+0.2 | - | - | - | +++ |
| 2.0+0.5 | - | - | - | +++ |
| **BA+IAA** | | | | |
| 0.5+0.1 | 40.81 | 5.25 | 3.15 | + |
| 0.5+0.2 | 30.59 | 3.50 | 2.50 | + |
| 0.5+0.5 | - | - | - | ++ |
| 1.0+0.1 | 50.63 | 4.50 | 3.25 | + |
| 1.0+0.2 | 55.33 | 6.35 | 3.10 | + |
| 1.0+0.5 | - | - | - | +++ |
| 2.0+0.1 | - | - | - | +++ |
| 2.0+0.2 | - | - | - | +++ |
| 2.0+0.5 | - | - | - | +++ |
| **BA+IBA** | | | | |
| 0.5+0.1 | 45.87 | 4.15 | 3.10 | + |
| 0.5+0.2 | 35.54 | 3.20 | 3.15 | + |
| 0.5+0.5 | - | - | - | ++ |
| 1.0+0.1 | 50.31 | 5.25 | 3.15 | + |
| 1.0+0.2 | 35.22 | 3.25 | 3.10 | + |
| 1.0+0.5 | - | - | - | +++ |
| 2.0+0.1 | - | - | - | +++ |
| 2.0+0.2 | - | - | - | +++ |
| 2.0+0.5 | - | - | - | +++ |
| **LSD at 5% level** | 6.66 | 1.12 | 0.84 | |

+, ++, +++, indicate slight, considerable, profuse callusing, respectively, -indicates no response.

### 3.3.3.2 Effect of Kn alone or in combination with NAA, IAA or IBA

This experiment was conducted with Kn singly or in combination with NAA, IAA or IBA on induction of adventitious shoots from *in vitro* grown internode explants in *S. rebaudiana.* Each treatment consisted of three replications and in each replication 12-15 explants were used. Data on percentage of explants showing shoot proliferation, number of shoots per culture and average length of the longest shoot were recorded after 5 weeks of culture and the results obtained are shown in Table 19.

From the results it was found that Kn alone failed to induce any adventitious shoots. Callus formation was observed in all the treatments. Generally the adventitious shoots developed from the induced callus but no relationship between amount of callus and number shoots was noted. While Kn with NAA were used the percentage of explants responded ranged from 0 to 60.52%, the number of shoots per culture ranged from 0 to 7.25 and the average length of the longest shoot ranged from 0 to 3.15 cm. In case of Kn with IAA, the percentage of explants responded ranges from 0 to 50.55%, the number of shoots per culture ranged from 0 to 5.10 and the average length of the longest shoot ranged from 0 to 3.15 cm. In case of Kn with IBA the percentage of explants responded ranges from 0 to 35.83%, the number of shoots per culture ranged from 0 to 3.40 and the average length of the longest shoot ranged from 0 to 2.65 cm. Among all the treatments the highest percentage of response 60.52% was recorded 1.0 mg/l Kn + 0.2 mg/l NAA followed by 55.90 with 1.0 mg/l Kn+0.1 mg/l NAA. Number of shoots per culture was also recorded highest in the same treatment of Kn + NAA. Of the three combinations Kn+NAA showed the best results.

So, from the above results it might be concluded that the combination of 1.0 mg/l Kn + 0.2 mg/l NAA was found to be the best combination for induction of adventitious shoots from *in vitro* grown internode explants in *S. rebaudiana.*

**Table 19. Effect of different concentration of Kn singly or in combination with NAA, IAA or IBA on induction of adventitious shoots from *in vitro* grown internode explants in *S. rebaudiana*. Each treatment consisted of 3 replications and in each replication 12-15 explants were used. Data were recorded after 5 weeks of culture.**

| Growth regulators (mg/l) | % of explants showing proliferation | No. of shoots per culture | Average length of the longest shoot (cm) | Callus formation |
|---|---|---|---|---|
| **Kn** | | | | |
| 0.5 | - | - | - | + |
| 1.0 | - | - | - | + |
| 2.0 | - | - | - | + |
| **Kn+NAA** | | | | |
| 0.5+0.1 | 35.87 | 4.30 | 3.15 | + |
| 0.5+0.2 | 30.35 | 4.10 | 3.10 | + |
| 0.5+0.5 | - | - | - | +++ |
| 1.0+0.1 | 55.90 | 6.15 | 2.90 | ++ |
| 1.0+0.2 | 60.52 | 7.25 | 1.15 | + |
| 1.0+0.5 | - | - | - | +++ |
| 2.0+0.1 | - | - | - | +++ |
| 2.0+0.2 | - | - | - | +++ |
| 2.0+0.5 | - | - | - | +++ |
| **Kn+IAA** | | | | |
| 0.5+0.1 | 30.83 | 3.90 | 3.15 | + |
| 0.5+0.2 | 25.21 | 3.20 | 2.75 | + |
| 0.5+0.5 | - | - | - | +++ |
| 1.0+0.1 | 50.55 | 5.10 | 3.00 | + |
| 1.0+0.2 | 40.39 | 3.95 | 3.15 | + |
| 1.0+0.5 | - | - | - | +++ |
| 2.0+0.1 | - | - | - | +++ |
| 2.0+0.2 | - | - | - | +++ |
| 2.0+0.5 | - | - | - | +++ |
| **Kn+IBA** | | | | |
| 0.5+0.1 | 25.12 | 3.15 | 2.50 | + |
| 0.5+0.2 | 20.25 | 2.35 | 2.65 | + |
| 0.5+0.5 | - | - | - | +++ |
| 1.0+0.1 | 30.71 | 3.25 | 2.10 | + |
| 1.0+0.2 | 35.83 | 3.40 | 2.10 | + |
| 1.0+0.5 | - | - | - | +++ |
| 2.0+0.1 | - | - | - | +++ |
| 2.0+0.2 | - | - | - | +++ |
| 2.0+0.5 | - | - | - | +++ |
| **LSD at 5 % level** | 4.80 | 0.58 | 0.90 | |

+, ++, +++, indicate slight, considerable, profuse callusing, respectively, -indicates no response.

### 3.3.4 Proliferation of adventitious shoot from leaf explants of *in vitro* grown shoots

#### 3.3.4.1 Effect of BA alone or in combination with NAA, IAA or IBA

In this experiment effect of different concentration of BA singly or in combination with NAA, IAA or IBA on induction of adventitious shoots from *in vitro* grown leaf explants in *S. rebaudiana* was studied. Each treatment consisted of three replications and in each replication 10-12 explants were used. Data on percentage of explants showing shoot proliferation, number of shoots per culture and average length of the longest shoot were recorded after 6 weeks of culture and the results obtained are shown in Table 20 (**Plate 16, Figs. A, B** and **C**).

From the results it was found that media with BA alone failed to induce any adventitious shoots. In BA+NAA four treatments, in BA+IAA four treatments and in BA+IBA three treatments the explants also failed to induce any adventitious shoots. The adventitious shoots developed along with the formation of callus on the cut site of the cultured explants. While BA with NAA were used the percentage of explants responded ranged from 0 to 90.36%, the number of shoots per culture ranged from 0 to 22.25 and the average length of the longest shoot ranged from 0 to 5.20 cm. In case of BA+IAA, the percentage of explants responded ranges from 0 to 70.52%, the number of shoots per culture ranged from 0 to 10.15 and the average length of the longest shoot ranged from 0 to 4.85 cm. Also in case of BA with IBA the percentage of explants responded ranged from 0 to 50.01%, the number of shoots per culture ranged from 0 to 8.85 and the average length of the longest shoot ranged from 0 to 4.35 cm. Among all the treatments the highest percentage of response 90.36% was recorded with 0.5 mg/l BA+0.1 mg/l NAA

followed by 75.56 with 1.0 mg/l BA+0.2 mg/l NAA. Number of shoots per culture was also recorded highest in the same treatment of BA + NAA.

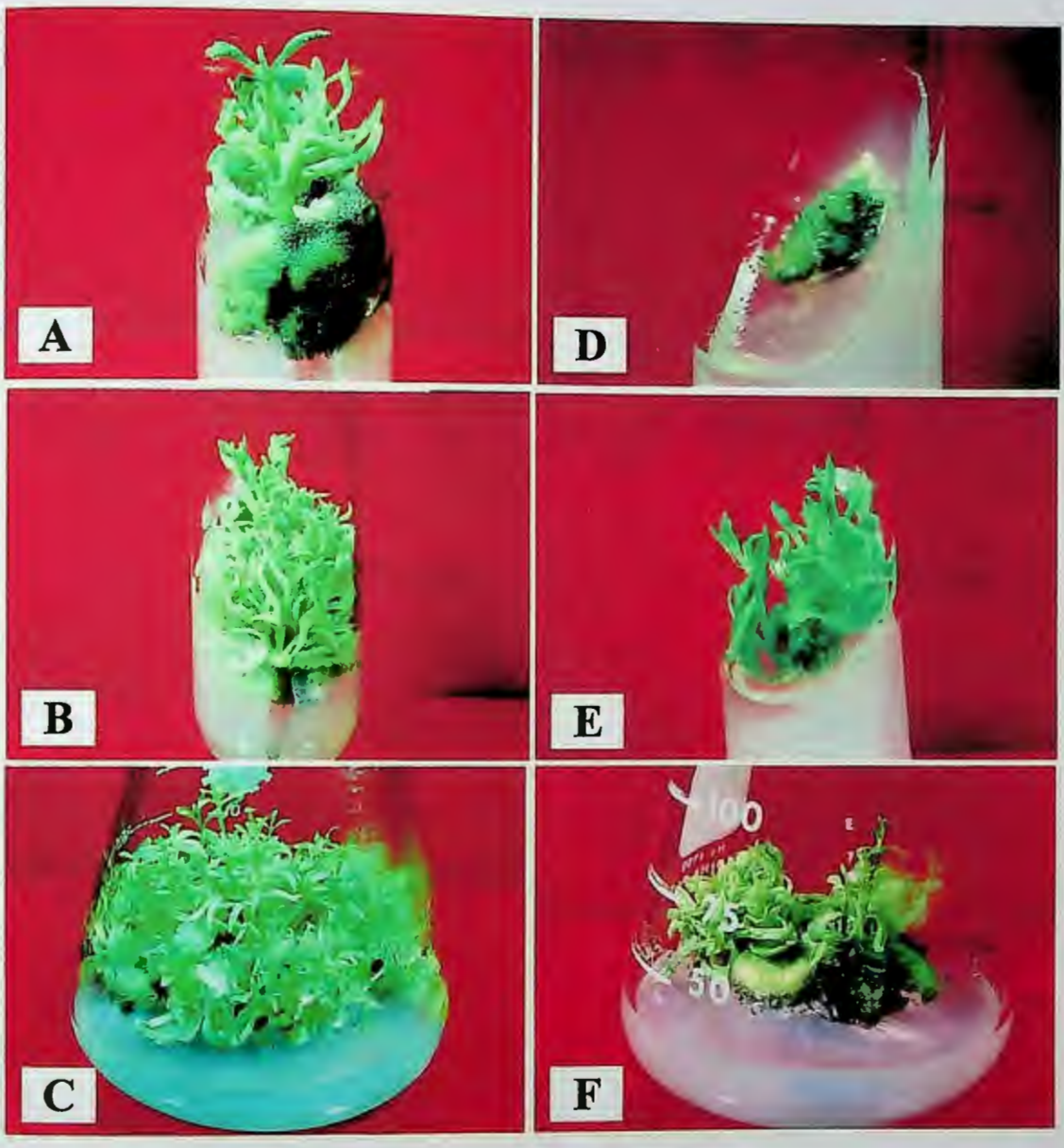
So, from the above results it might be concluded that the combination of 0.5 mg/l BA+0.1 mg/l NAA was found to be the best combination for induction of adventitious shoots from *in vitro* grown leaf explants in *S. rebaudiana* like internode exolants, leaf explants also showed better results in BA+NAA combination.

**Table 20. Effect of different concentration of BA singly or in combination with NAA, IAA or IBA on induction of adventitious shoots from *in vitro* grown leaf explants *S. rebaudiana*. Each treatment consisted of 3 replications and in each replication 10-12 explants were used. Data were recorded after 6 weeks of culture.**

| Growth regulators (mg/l) | % of explants showing proliferation | No. of shoots per culture | Average length of the longest shoot (cm) | Callus formation |
|---|---|---|---|---|
| **BA** | | | | |
| 0.5 | - | - | - | + |
| 1.0 | - | - | - | + |
| 2.0 | - | - | - | + |
| **BA+NAA** | | | | |
| 0.5+0.1 | 90.36 | 22.25 | 5.20 | + |
| 0.5+0.2 | 75.56 | 10.15 | 4.55 | ++ |
| 0.5+0.5 | - | - | - | +++ |
| 1.0+0.1 | 70.21 | 13.50 | 4.80 | + |
| 1.0+0.2 | 80.28 | 16.39 | 4.90 | + |
| 1.0+0.5 | 50.12 | 7.20 | 3.95 | +++ |
| 2.0+0.1 | - | - | - | +++ |
| 2.0+0.2 | - | - | - | +++ |
| 2.0+0.5 | - | - | - | +++ |
| **BA+IAA** | | | | |
| 0.5+0.1 | 55.36 | 5.15 | 3.50 | + |
| 0.5+0.2 | 45.42 | 4.65 | 3.25 | + |
| 0.5+0.5 | - | - | - | ++ |
| 1.0+0.1 | 70.52 | 10.15 | 4.85 | + |
| 1.0+0.2 | 60.68 | 8.35 | 4.45 | + |
| 1.0+0.5 | 45.71 | 4.40 | 3.15 | ++ |
| 2.0+0.1 | - | - | - | +++ |
| 2.0+0.2 | - | - | - | +++ |
| 2.0+0.5 | - | - | - | +++ |
| **BA+IBA** | | | | |
| 0.5+0.1 | 40.12 | 5.20 | 3.35 | + |
| 0.5+0.2 | 35.32 | 4.25 | 3.20 | + |
| 0.5+0.5 | - | - | - | ++ |
| 1.0+0.1 | 45.42 | 5.35 | 4.10 | + |
| 1.0+0.2 | 50.01 | 8.85 | 4.35 | + |
| 1.0+0.5 | 30.09 | 3.15 | 3.10 | ++ |
| 2.0+0.1 | 15.36 | 3.10 | 2.80 | ++ |
| 2.0+0.2 | - | - | - | +++ |
| 2.0+0.5 | - | - | - | +++ |
| **LSD at 5% level** | 6.00 | 1.31 | 0.95 | |

+, ++, +++, indicate slight, considerable, profuse callusing, respectively, -indicates no response.

# PLATE-16




Growth and development of adventitious shoots of *Stevia rebaudiana* from leaf explants on MS + 0.5 mg/l BA + 0.1 mg/l NAA after 3 weeks (**Fig. A**), after 4 weeks (**Fig. B**), after 6 weeks (**Fig. C**) and from internode explants on 1.0 mg/l Kn + 0.2 mg/l NAA after 3 weeks (**Fig. D**), after 4 weeks (**Fig. E**) and after 6 weeks (**Fig. F**) of growth.

### 3.3.4.2 Effect of Kn alone or in combination with NAA, IAA or IBA

In this experiment effect of different concentration of Kn singly or in combination with NAA, IAA or IBA on induction of adventitious shoots from *in vitro* grown leaf explants in *S. rebaudiana* was studied. Same concentrations were used as in previous one. Each treatment consisted of three replications and in each replication 8-10 explants were used. Data on percentage of explants showing proliferation, number of shoots per culture and average length of the longest shoot were recorded after 5 weeks of culture and the results obtained are shown in Table 21 and **Plate 16 (Figs. D, E** and **F)**.

From the results it was found that Kn alone did not formed any adventitious shoots. While Kn with NAA was used the percentage of explants responded ranged from 0 to 60.00%, the number of shoots per culture ranged from 0 to 10.15 and the average length of the longest shoot ranged from 0 to 4.19 cm. In case of Kn with IAA, the percentage of explants responded ranged from 0 to 40.13%, the number of shoots per culture ranged from 0 to 6.25 and the average length of the longest shoot ranged from 0 to 3.19 cm. In case of Kn with IBA the percentage of explants responded ranged from 0 to 35.69%, the number of shoots per culture ranged from 0 to 4.51 and the average length of the longest shoot ranged from 0 to 2.75 cm. Among all the treatments the highest percentage of response 60.00% was recorded 1.0 mg/l Kn + 0.2 mg/l NAA followed by 50.02% with 1.0 mg/l Kn+0.1 mg/l NAA. Kn+NAA combination showed better performances than other two combinations.

So, from the above results it might be concluded that the combination of 1.0 mg/l Kn + 0.2 mg/l NAA was found to be the best combination for induction of adventitious shoots from *in vitro* grown leaf explants in *S. rebaudiana*.

**Table 21. Effect of different concentration of Kn singly or in combination with NAA, IAA or IBA on induction of adventitious shoots from *in vitro* grown leaf explants in *S. rebaudiana*. Each treatment consisted of 3 replications and in each replication 8-10 explants were used. Data were recorded after 5 weeks of culture.**

| Growth regulators (mg/l) | % of explants showing proliferation | No. of shoots per culture | Average length of the longest shoot (cm) | Callus formation |
|---|---|---|---|---|
| **Kn** | | | | |
| 0.5 | - | - | - | + |
| 1.0 | - | - | - | + |
| 2.0 | - | - | - | + |
| **Kn+NAA** | | | | |
| 0.5+0.1 | 45.35 | 6.35 | 3.32 | + |
| 0.5+0.2 | 35.12 | 5.21 | 3.25 | + |
| 0.5+0.5 | - | - | - | ++ |
| 1.0+0.1 | 50.02 | 8.25 | 3.91 | ++ |
| 1.0+0.2 | 60.00 | 10.15 | 4.19 | + |
| 1.0+0.5 | 30.36 | 4.39 | 3.12 | ++ |
| 2.0+0.1 | - | - | - | +++ |
| 2.0+0.2 | - | - | - | +++ |
| 2.0+0.5 | - | - | - | +++ |
| **Kn+IAA** | | | | |
| 0.5+0.1 | 30.96 | 4.94 | 3.15 | + |
| 0.5+0.2 | 25.58 | 3.75 | 2.93 | + |
| 0.5+0.5 | - | - | - | +++ |
| 1.0+0.1 | 35.64 | 5.26 | 3.15 | + |
| 1.0+0.2 | 40.13 | 6.25 | 3.19 | + |
| 1.0+0.5 | 30.25 | 3.65 | 2.42 | ++ |
| 2.0+0.1 | - | - | - | +++ |
| 2.0+0.2 | - | - | - | +++ |
| 2.0+0.5 | - | - | - | +++ |
| **Kn+IBA** | | | | |
| 0.5+0.1 | 35.69 | 4.51 | 2.45 | + |
| 0.5+0.2 | 20.35 | 2.15 | 2.51 | + |
| 0.5+0.5 | - | - | - | +++ |
| 1.0+0.1 | 25.91 | 2.65 | 2.75 | + |
| 1.0+0.2 | 30.19 | 3.35 | 2.12 | + |
| 1.0+0.5 | 20.25 | 2.52 | 1.85 | ++ |
| 2.0+0.1 | - | - | - | +++ |
| 2.0+0.2 | - | - | - | +++ |
| 2.0+0.5 | - | - | - | +++ |
| **LSD at 5% level** | **3.80** | **0.44** | **0.74** | |

+, ++, +++, indicate slight, considerable, profuse callusing, respectively, -indicates no response.

### 3.3.5 Rooting of microcuttings

The induced shoots were cultured in root inducing medium for adventitious rooting to raise full-fledged plants. In this experiment effect of different concentration of IBA, NAA or IAA in $MMS_1$ medium on adventitious root formation from microcuttings in *S. rebaudiana* was studied. In each type of auxin four different concentrations (0.1, 0.2, 0.5 and 1.0 mg/l) were tested. Each treatment consisted of three replications and in each replication 10-12 explants were used. Data on percentage of explants showing proliferation, number of shoots per culture and average length of the longest shoot were recorded after 5 weeks of culture initiation and the results obtained are shown in Table 22 and **Plate 17 (Figs. A, B and C).**

From the results it was found that media with no auxin the microcuttings failed to induce any root but in all other treatments the explants formed roots. When IBA singly was used the percentage of cuttings rooted responded ranged from 65.38 to 97.66%, the number of roots per cutting ranged from 3.15 to 12.10 and the average length of the longest root ranged from 2.12 to 3.05 cm. In case NAA singly was used the percentage of cuttings rooted responded ranged from 75.56 to 95.13%, the number of root per cutting ranged from 3.10 to 7.35 and the average length of the longest root ranged from 2.38 to 2.95 cm. When IAA singly was used the percentage of cuttings rooted ranged from 55.99 to 75.55%, the number of roots per cutting ranged from 2.85 to 3.65 and the average length of the longest root ranged from 2.93 to 3.65 cm. Among all the treatments the highest percentage of response 97.66% was recorded in $MMS_1$ with 0.2 mg/l IBA followed by 95.13 with 0.2 mg/l NAA.

On average, among different concentrations of auxin the highest percentage of explants showing root proliferation was obtained in 0.2 mg/l and among three auxins NAA and IBA showed equal response in terms of frequency but number of roots was maximum in IBA.

From the above results it might be concluded that $MMS_1$ media with single IBA of 0.2 mg/l was found to be the best combination on adventitious root formation from microcuttings in *S. rebaudiana*. Same concentrtation of NAA was also equally effective.

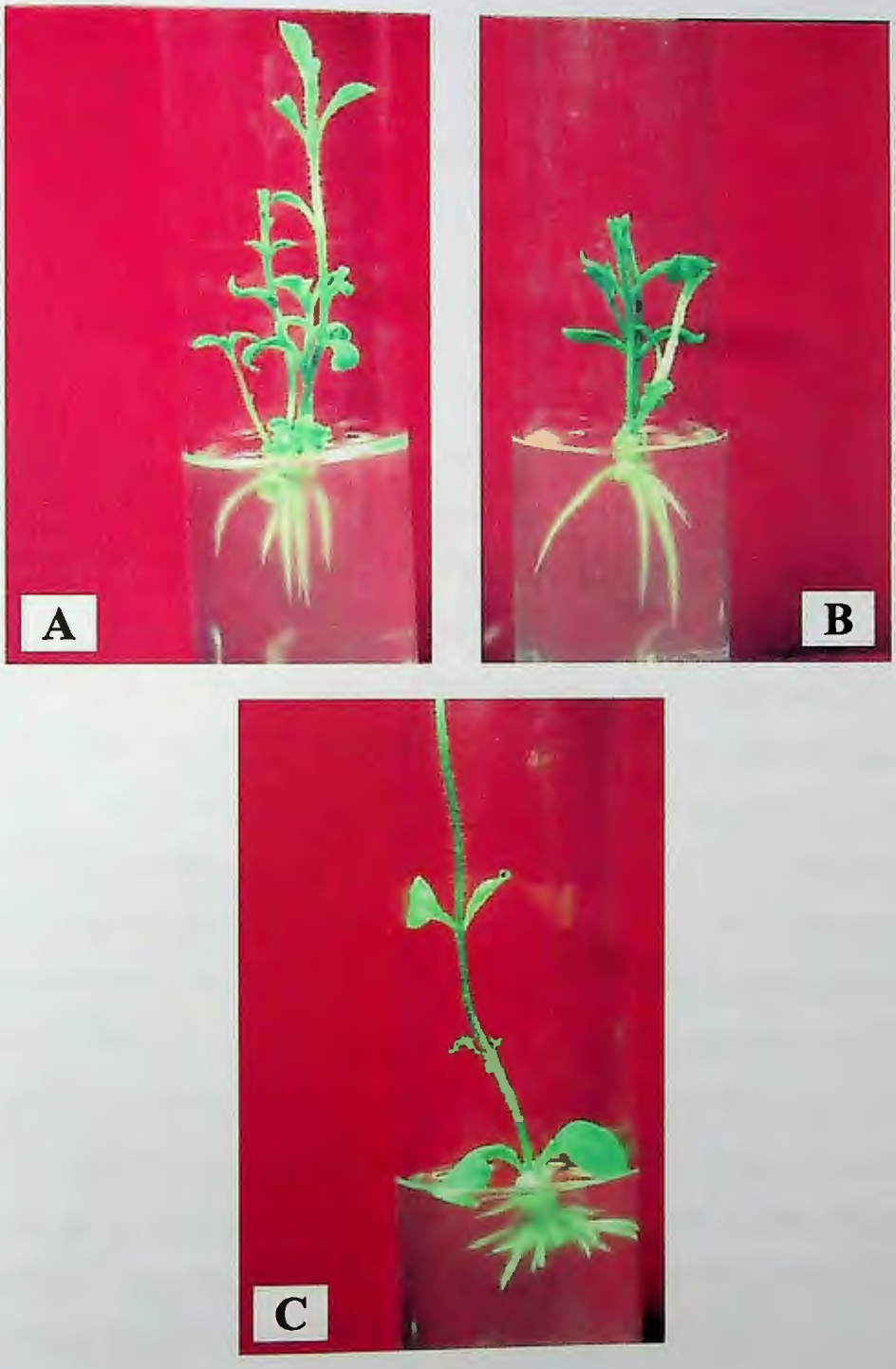
**Table 22. Effect of IBA, NAA or IAA in $MMS_1$ medium on adventitious root formation from microcuttings in *S. rebaudiana*. Each treatment consisted of 3 replications and in each replication 10-12 explants were used. Data were recorded after 5 weeks of culture.**

| Type of auxin | Conc. of auxin mg/l | % of cuttings rooted | No. of roots per cutting | Average length of the longest root (cm) | Days to mergence of roots | Callus formation at the cutting base |
|---|---|---|---|---|---|---|
| Nil | - | - | - | - | - | - |
| IBA | 0.1 | 92.37 | 6.35 | 2.23 | 6-14 | - |
| | 0.2 | 97.66 | 12.10 | 2.12 | 5-12 | + |
| | 0.5 | 85.11 | 4.25 | 2.88 | 7-12 | + |
| | 1.0 | 65.38 | 3.15 | 3.05 | 6-15 | ++ |
| **Mean** | | **85.13** | **6.46** | **2.57** | | |
| NAA | 0.1 | 91.55 | 5.55 | 2.55 | 7-12 | + |
| | 0.2 | 95.13 | 7.35 | 2.38 | 7-12 | + |
| | 0.5 | 80.88 | 3.30 | 2.95 | 7-12 | ++ |
| | 1.0 | 75.56 | 3.10 | 2.52 | 7-12 | ++ |
| **Mean** | | **85.78** | **4.83** | **2.60** | | |
| IAA | 0.1 | 55.99 | 2.85 | 3.01 | 8-15 | - |
| | 0.2 | 70.72 | 3.50 | 3.65 | 8-12 | - |
| | 0.5 | 75.55 | 3.65 | 2.93 | 8-12 | + |
| | 1.0 | 60.43 | 3.20 | 3.55 | 8-14 | ++ |
| **Mean** | | **65.67** | **3.30** | **3.29** | | |
| **Effect of Phytohormone[1]** | | | | | | |
| IBA | | 85.13a | 6.46a | 2.57b | | |
| NAA | | 85.78a | 4.83b | 2.60b | | |
| IAA | | 65.67b | 3.30c | 3.29a | | |
| **Effect of concentration[2]** | | | | | | |
| 0.1 mg/l | | 79.97c | 4.92b | 2.60b | | |
| 0.2 mg/l | | 87.84a | 7.65a | 2.72b | | |
| 0.5 mg/l | | 80.51b | 3.73c | 2.73b | | |
| 1.0 mg/l | | 67.02d | 3.15c | 3.04a | | |

[1]Means of three replications and four kinds of concentration
[2]Means of three replications and three kinds of phytohormone
+, ++, +++, indicate slight, considerable, profuse callusing, respectively,
- indicates no response.

Comparison between means of different characters was made using Duncan's multiple range test; the values in each column carrying different letters are significantly different at 5% level of probability.

# PLATE-17

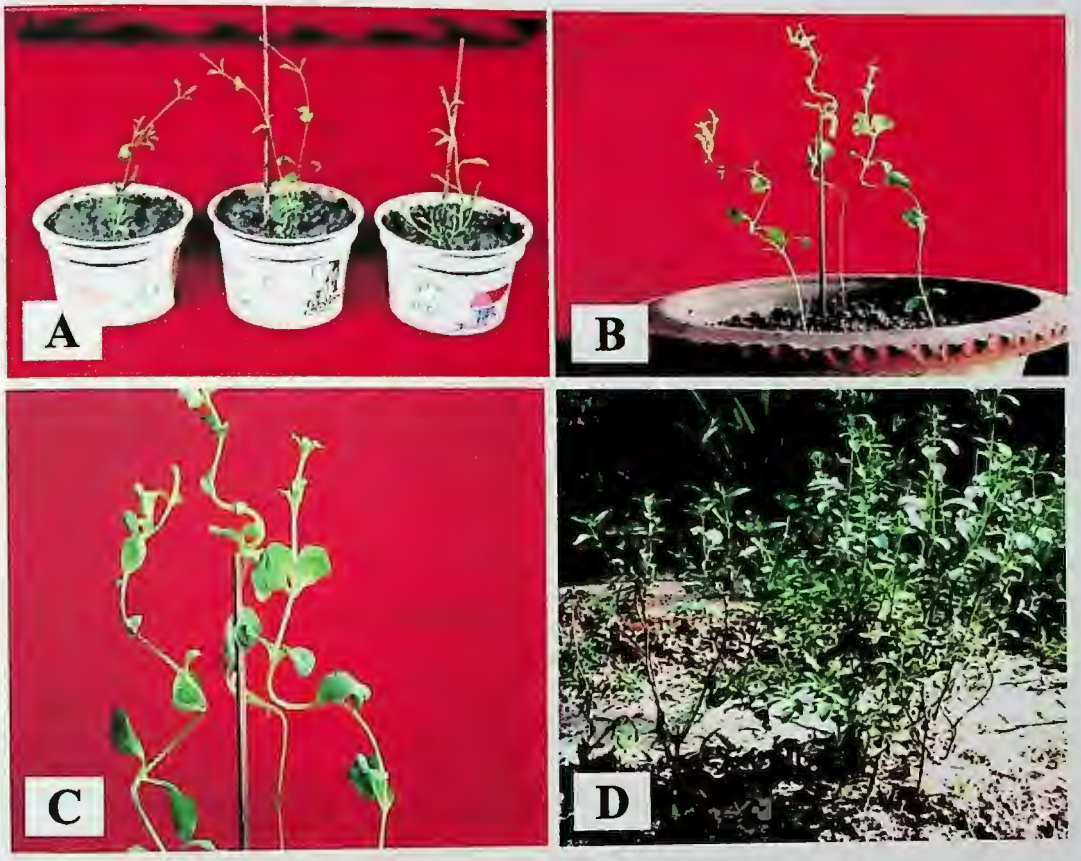



Adventitious root formation from microcuttings on $MMS_1$ medium with 0.2 mg/l IBA (**Fig. A**), 0.2 mg/l NAA (**Fig. B**) and 0.5 mg/l IAA (**Fig. C**)

### 3.3.6 Acclimatization and establishment of *in vitro* regenerated plantlets on soil

When the regenerated shoots developed extensive root system they were needed to be transferred in soil. Before transplantation the individual regenerated plantlet was brought out of the culture medium and its root system was made agar gel free by continuous flowing of tap water. Then they were washed with 0.35% diatheim-45 (Fungicide). The plantlets were then ready for transplantation. A good transplantation always requires some conditions. These are preparation of soil, sterilization of soil, and supply of moisture and ventilation. Therefore, the rooted microcuttings (plantlets) were initially planted in plastic pots containing compost and soil (1:1). Each and every newly potted plants in plastic pots were then watered adequately, covered with a perforated polythene bag and kept in growth chamber for 10-15 days so that the plants adapts themselves to the soil environment. After a few days of indoor acclimatization, the plantlets from plastic pots were transplanted directly to the field or to large pots. Plants were initially established in plastic pots with a view to easy handling during transplantation to the field. It was observed that the previling atmospheric condition (mostly, humidity and temperature) of transplanting season greatly influenced the initial survival of potted plants. The plantlets having 2-4 cm roots at their active elongation period survived better than those transferred with much elongated and branched root systems. Although many regenerates were transplanted only 65% among them survived and could be acclimatized successfully. In natural habitat *in vitro* plantlets of *Stevia rebaudiana* was grown very healthy, after 6 months its height was about 1 meter long with many branches **(Plate 18, Figs. A, B, C** and **D)**.

# PLATE-18




Growth of transplants of *Stevia rebaudiana* on compost and soil (1:1) after 2 weeks (**Fig. A**), on garden soil after 4 weeks (**Fig. B**), close up view (**Fig. C**) and acclimatized under natural environment (**Fig. D**) after 6 months.

# CHAPTER 2

# DISCUSSION

The regeneration of plants from cultured tissues or plant parts is a key stem in the application of tissue culture methodology for plant propagation and conservation. The development of efficient protocols for reproducible high frequency of plant regeneration from cultured tissue would therefore assume great importance. To achieve high frequency of plant regeneration selection of appropriate explants is a primary prerequisite. Explants must generally be chosen from healthy vigorously growing plants to obtain optimum results.

The initiation of organs *in vitro* is a complex morphogenic phenomenon, in which extrinsic and intrinsic factors play a role. *In vitro* mass propagation of plant species is one of the best and most successful examples of commercial application of plant tissue culture technology. This is particularly important in the propagation and management of plant species, which has immense commercial potentiality. Recently there has been much progress in this field of biotechnology. Importance of tissue culture of useful plants in propagation, conservation of genetic resources and clonal improvement has been described by many authors (Barz *et al.*, 1977; Data and Datta, 1985; Kukreja *et al.*, 1989).

## 4.1. *A. vera*

*Aloe vera* L. is a xerophytic perennial herb with a rosette of long thick lanceolate leaves with spiny margins. *Aloe vera* plant originated in the warm, dry climates of Africa. In the United States, it is grown commercially in the Rio Grande Valley of Texas, in California and Florida, and in specially designed Green Houses in Oklahoma State. It is an important medicinal plant and leaves of *Aloe vera* are anthraquinone glycosides, called aloins, which include barbaloin, iso-barbaloin, β-barbaloin, emodin, chrysophanol, chrysophanic acid

and aloe-emodin, uronic acid and enzymes. Resins, sterols, triterpenes, cuomarins, saponins, carbohydrates, amino acids and vitamins are also present in this valuable plant. Mass propagation of *A. vera* through *in vitro* culture was standardized in the first part of this investigation for commercial application and conservation.

Different experiments were conducted to optimize culture condition for establishment of shoot cultures from shoot tip explants of field grown *A.vera* plant and further multiplication of shoots from the *in vitro* grown explants. Among different strengths of MS medium with two phytohormone treatments full MS media with 0.5 mg/l BA was found to be the best treatment for primary establishment shoot cultures from shoots tip explants collected from field grown plants of *A. Vera*. Combinations of BA and Kn were also effective for multiple shoot development in *Aloe vera* explants. The highest percentage of response 90.71% was recorded in MS with 2.0 mg/l BA and 75.93 with 0.5 mg/l Kn.

In the third experiment, out of fifteen combinations of BA with Kin the highest percentage of explants showing shoots proliferation and maximum number of shoots per culture were recorded 90.51% and 10.46 respectively, which were found in media with 2.0 mg/l BA+0.5 mg/l Kn. Different concentrations of BA with NAA and IAA on shoot proliferation from *in vitro* grown shoot tip explants of *A. vera* was successfully studied and the highest percentage of response and number of shoots per culture were recorded 95.30% and 12.43 in MS with 2.0 mg/l BA+0.2 mg/l NAA. The combined effect of BA, Kn and NAA on multiple shoot regeneration from shoot tip explants of *in vitro* regenerated shoot was also studied on MS medium. Among the different experiments so far conducted best response towards multiple shoots regeneration was obtained when MS medium was supplemented with 2.0 mg/l BA + 0.5 mg/l Kn + 0.2 mg/l NAA. In this experiment frequency of shoottip explants produced multiple shoots was recorded 98.96 % and highest number of shoots per culture was 15.39.

The early sprouting and rapid growth of shoots were noticed on medium with lower agar concentrations (2-4 mg/l). The easy availability of nutrient elements like Ca, Mg, K and Mn could be distributed in the soft-gel medium (Debergh, 1983). *In vitro* growth abnormalities like fascinated shoots and vitrified leaves have also been observed in guava cultures grown with lower concentrations of agar (Amin and Jaiswal, 1989). This growth anomalies are apprehended to be the changes in the matric potential of media water under lower gelling agent concentrations. The highest percentage of explants showing shoot proliferation was noticed 90.54% in Merck Ltd., Mumbai, India; 95.32% of *Phyto* Technology Laboratories, USA and 95.56% in BDH chemicals Ltd. Poole, England, UK the all the best results were observed with 7 g/l of agar. Reduced growth and less number of shoots on media gelled with 10 gm/l agar (above all grades) could be due to restricted diffusion of macronutrients (Romberger and Tabor, 1971) or reduced availabilit of organic matter and water (Skirvin, 1981 and Debergh, 1983). It is evident from the results of present investigation and also those of others (Debergh, 1983; Amin and Jaiswal, 1989) that concentration of agar in the media can affect the culture growth in many ways. Therefore, the level of agar in the medium should be such that it minimizes the water loss and allows the good diffusion of nutrient elements.

Sucrose is usually the source of carbon and energy. It has considerable influence on growth and morphogenesis in culture media (Fadia and Metha, 1973; Maeda, 1980). The type and concentration of sugar is not the only factor that promote both germination and growth of seedlings but also promote growth under *in vitro* condition (Liddell, 1953; Karasawa, 1966; Anderson, 1967; Ernst, 1967; Arditti *et. al.* 1972). A marked difference in the rate of shoot formation was observed in the range and quality of sucrose concentrations. The highest percentage of explants showing shoot proliferation was recorded 85.05% in Merck Ltd., Mumbai; 99.02% in *Phyto* Technology Laboratories, USA, 99.22% in BDH Chemicals Ltd., England and 82.71% in local market sugar. Except local market sugar satisfactory the results in all sources were

observed with 30 g/l of sucrose. BDH Chemicals Ltd., England showed the best results for shoot proliferation from *in vitro* grown shoot tip explants of *A. Vera* in medium with 2.0 mg/l BA+0.5 mg/l Kn+0.2 mg/l NAA. A concentration of 5% sucrose was found optimum for citrus tissue culture (Murashige and Tucker, 1969). Miah *et al.* (1986) reported shoot differentiation in rice using 7% sucrose in MS medium. Inden *et al.* (1988) reported good response to shoot regeneration in 2.0% sucrose containing MS medium in ginger. In the present investigation lower concentration 2.0% sucrose and higher concentration 5.0% sucrose have been proved less efficient for shoot proliferation i.e. shoot size and number decrease and root growth was inhibited. Yoshiji and Onozawa (1997) reported similar results that at 8.0% and 12.0% sucrose concentration reduced shoot growth in *Lilium rubellum.*

Most plants exhibit varied degrees of responses to medium, the herbaceous and semi-woody species respond better than the woody ones (Bhojwani and Razdam, 1983). The herbaceous nature of *Aloe vera* indicates its suitability to culture on MS medium. Many authors reported that several medicinal plant species nicely responded on MS medium e.g. *Asclepias curassavica* (Pramanic and Datta, 1986); *Adhatoda vasica* (Amin *et al.* 1997); *Rauvolfia serpentina* (Roja *et al.* 1990; Illahi and Akram 1987, Ahmad *et al.* 2002), *Ixora fulgens* (Amin *et al.* 2002); *Plantago ovata* (Barna and Wakhlu, 1988); *Plumbago rosea* (Kumar and Bhavanandan, 1988); *Rheum emodi* (Lal and Ahuja, 1989); *Chrysanthemum morifolium* (Karim *et al.*2003); *Solanum torvum* (Jaiswal and Naragan, 1985); *Aloe vera* (Hirimburegama and Gamage, 1995, Wang *et al.* 2002, Zhou *et al.* 1999, Roy and Sarkar 1991, Gui *et al.* 1990). *In vitro* plant regeneration depends on a number of factors including the composition of culture medium, proper concentration of growth regulators, and the response of the explants as well as the genotype of the plant material. Commonly, it has been shown that the basic regulatory mechanism underlying plant organ initiation involves a balance between auxin and cytokinin. A relatively low level of auxin and high level of cytokinin results in shoot initiation, whereas the reverse condition results in root formation. Superiority

of BA over other cytokinins in producing *in vitro* shoots has also been confirmed in other plants like *Rosmarinus officinalis* (Misra and Chaturavedi, 1984); *Arachis hypogaea* (Mhatre *et al.* 1985); *Camellia sinensis* (Arulpragasam and Latif, 1986); *Atropa belladonna* (Benjamin *et al.* 1987) and *Adhatoda vasica* (Amin *et al.* 1997). Wareing and Phillips (1981) showed that synthetic cytokinin such as BA was more active than naturally occurring cytokinin in shoot proliferation. Present experiments also support this agreement. Bhagyalakshmi and Sing (1988) also observed increased multiple shoot formation with poor shoot elongation and root formation using BA and Kn together. Another experiment was conducted with different concentration and combination of cytokinin and auxin. Among different combinations BA and NAA combination was proved efficient in shoot proliferation. Gui *et al.* (1990) als obtained good response towards multiple shoot regeneration with 2 ppm zeatin plus 0.5 ppm NAA in *A. vera*.

Several reports are available on MS medium with different concentrations and combinations of BA and NAA for multiple shoot regeneration from different explants of ginger (Ikeda and Tanaka, 1989; Bhagyalakshmi and Shing, 1988; Sunitibala *et al.* 2001). Results of the present study show that BA and NAA combination was better than BA-IBA, BA-IAA, Kn-NAA, Kn-IBA, Kn-IAA combinations. Possitive morphogenic potentially and plant regeneration in presence of BA and Kn or 2ip was also reported by Amin *et al.* 1992 and Islam *et al.* 1997.

Shoot development as well as rooting of regenerated shoots is especially important for establishing tissue culture derived plantlets (Moss *et al.*, 1988). Adventitious root formation can be induced quite readily in many herbaceous species but not in most woody species (Bajaj, 1991). In the present study it was observed that herbaceous *Aloe vera*, a monocotyledons plant could not be rooted easily. Thus an experiment was conducted on modified MS media with or without auxins for proper root induction. Most plants required the presence

of auxins for root induction. The importance of auxin in root induction was first reported by Gautheret (1945). Auxins are regarded as the main factor promoting root initiation (Tizio *et al.*, 1968; Lee Chong *et al.*, 1969). For induction of roots, the regenerated microshoots were separated from the shoot cultures and planted on $MMS_1$ basal salt medium supplemented with different auxins singly. The highest percentage of response 80.25% was recorded in $MMS_1$ with 0.2 mg/l NAA followed by 60.85% with 0.2 mg/l IBA. Number of roots per microshoot and length of the longest root were also recorded highest in the same concentration. After producing sufficient number of roots, the plantlets needed to be transplanted into potted soil and finally to the out door condition through gradual acclimatization. Rooted plantlets with 3-4 well-developed leaves were brought out of the culture vessels. Their roots were washed thoroughly by running tap water to remove the sticky agar and to prevent unwanted fungal or bacterial growth in the roots. The plantlets were transferred on to the small plastic potted soil. Pierik (1987) reported that the most effective auxins were definitely NAA and IBA for rooting. There have been several published reports on the induction of roots by auxin in regenerated shoots of different monocotyledonous plants (Zaid and Hughes, 1995; Khatun *et al.*, 1997; Azad and Amin, 1999; Rabbani and Ali, 1999; Roy *et al.*, 1999 and Mohammad *et al.*, 2000). In the monocotyledons Novak *et al.*, (1986) reported that plantlets were successfully transplanted in to perlite saturated with hormone and sucrose free $^1/_2$ MS and cultured in moisture growth chamber (glass house) for two weeks. In this study 70% survival was recorded after transfer of *in vitro* raised plants to *ex vitro* condition.

### 4.2. *A. calamus*

Sweetflag (*Acorus calamus* L.) is a rare aromatic plant of immense ethno-botanical uses. So far no tissue culture work has been conducted on this important medicinal plant in Bangladesh. Therefore, the present study was undertaken to establish reproducible protocols for large-scale micropropagation

of the experimental plant. The present investigation was designed to conduct experiments using the explants from both the mature field grown plants and the *in vitro* grown plantlets. Results of the present study are discussed in the following paragraphs. In the present investigation, MS medium with various hormonal supplements was used for optimizing multiple shoot regeneration protocol from rhizome tip explants. Between the two types of cytokinin (BA and Kn) used the highest percentage of response 95.86% was recorded with 3.0 mg/l BA and 90.11% with 2.0 mg/l Kn. Number of shoots per culture was also recorded highest in the same treatments. The best response of shoot proliferation was obtained with 3.0 mg/l concentration of BA.

Further experiments were conducted for rapid multiplication of shoots from the primary established shoot cultures. Different concentrations of BA and Kn singly and combinedly were used for multiple shoot formation. Nine combinations were tested for the above purpose. The highest percentage of response 61.66% was recorded with 2.0 mg/l BA, 45.32% with 2.0 mg/l Kn and 66.66% with 1.0 mg/l BA+1.0 mg/l Kn. Average length of longest shoot per culture was also recorded highest in the same concentration and combination. So MS media with 1.0 mg/l of BA+1.0 mg/l Kn was found to be the best for multiple shoot proliferation from *in vitro* grown shoot tip explants of *A. calamus*. Sometimes media with different concentrations of a cytokine with an auxin on shoot proliferation are also effective. Therefore an experiment was conducted with BA with NAA or IAA for shoot proliferation in *A.calamus*. The highest percentage of response 98.90% was recorded with 2.0 mg/l BA+0.1 mg/l NAA followed by 95.18% with 2.0 mg/l BA+0.5 mg/l NAA. Number of shoots per culture was also recorded highest in the same combination. Similarly the combined effect of Kn with NAA or IAA on multiple shoot development from the *in vitro* regenerated shoot tips was also studied in the present investigation on MS medium. The highest percentage of response 98.99% was recorded with 2.0 mg/l Kn + 0.05 mg/l NAA followed by 97.33% with same

combination of Kn+IAA. Number of shoots per culture was also recorded highest in the same combination. Other factors like sucrose also affect *in vtiro* growth and proliferation of shoots. Sucrose has considerable influence on growth and morphogenesis of cultured explants in culture media (Fadia and Metha, 1973; Maeda, 1980). In the present investigation the highest percentage of explants showing shoot proliferation was noticed 97.93% in Merck Ltd., Mumbai; 93.33% in *Phyto* Technology Laboratories, USA, 86.67% in BDH Chemicals Ltd., England and 93.34% in local market sugar. In all cases of sugar brands the best results were observed with 40 g/l of sucrose. So 40 g/l sucrose of Merck Ltd., Mumbai was considered as the best for multiple shoot proliferation from shoot tip explants of *in vitro* proliferated shoots of *A. calamus* with BA 2.0 mg/l + NAA 0.5 mg/l. Cytokinin and auxin in various concentrations and combinations were also tested in this investigation. BA with NAA or IAA was tested to select the optimum combination and concentration of the required hormone for multiple shoot induction and BA and NAA combination was proved to be efficient for shoot proliferation. Inden *et al.* (1988) also obtained good response in multiple shoot regeneration of ginger using same hormonal supplements in MS medium. The growth and development of regenerated shoots obtained by Inden *et al.* (1988) were healthy and faster. Reghunath (1989) was also able to regenerate multiple shoots in cardamom using the combinations of BA and NAA in MS medium. Malamug *et al.* (1991) was also able to proliferate multiple shoots from the *in vitro* regenerated shoots in ginger using BA and NAA in modified medium.

The type and concentrations of sugar not only promote germination and growth of seedlings but also affect *in vitro* growth and development of cultured explants.(Liddell, 1953; Karasawa, 1966; Anderson, 1967; Ernst, 1967; Arditti *et al.*, 1972). The explants formed shoots but different parameters differed according to type and level of sucrose. The highest percentages of explants showing shoot proliferation were noticed 97.93% in Merck Ltd., Mumbai;

93.33% in *Phyto* Technology Laboratories, USA, 86.67% in BDH chemicals Ltd., England and 93.34% in local market sugar. In all cases best result was obtained at 40 g/l of sucrose.

Rooting of the *in vitro* regenerated shoots is especially important for establishing tissue culture derived plantlets in the *ex vitro* condition (Moss *et al.*1988). Most plants require the presence of auxin for root induction. Gautheret (1945) first suggested the importance of auxin in root induction. Different concentration and combination of NAA and IBA singly or combinedly on *in vitro* root formation from microcuttings in *A. calamus* was studied. The highest percentage of response 40.51% was recorded with 1.0 mg/l IBA and number of root per cutting and length of the longest root was also recorded highest in the same concentration. More or less similar responses were obtained by Balachandran *et al.*, (1990) and Hoque *et al.*, (1999). The rooted plantlets were successfully acclimatized on the soil under natural condition and about 68% of them survived.

### 4.3. *S. rebaudiana*

*Stevia*, a natural sweetener plant that contains stevioside which is considered 300 times sweeter than sucrose at 0.4% sucrose and 100 times sweeter at 10% sucrose concentration. It is native to the valley of the Rio Monday in highland of Paragauy and now it is introduced as a new medicinal plant in Bangladesh.

*In vitro* mass propagation of plant species is one of the best and most successful examples of commercial application of plant tissue culture technology. This is particularly important in the propagation and management of plant species like *Stevia rebaudiana.* Importance of tissue culture of useful plants in propagation and conservation of genetic resources and clonal improvement has been described by many authors (Barz *et la.*, 1977; Data and Datta, 1985; Kukreja *et al.*, 1989). The present investigation was designed to conduct

experiments using explants from pot grown plants of *S. rebaudiana* for mass propagation and conservation. The collected explants were capable of producing multiple shoots *in vitro* and subsequently complete plantlets by producing roots on the shoot cuttings. However, different responses and results were obtained from different and treatments. Possible causes of such differences have been discussed in the following paragraphs. In the first experiment effect of different concentrations of BA with Kn singly or combinedly on shoot proliferation from nodal explants of pot growing plants was studied. The highest percentage of response, number of shoots per culture and average length of longest shoot per culture were 73.33%, 4.50 and 3.50 cm respectively was recorded with 1.5 mg/l BA+1.0 mg/l Kn. After establishment of primary shoot cultures from shoot tip explants of pot growing *S. rebaudiana* plants further multiplication of shoots was done using nodal segments of *in vitro* grown shoots. For regeneration of shoots from nodal explants BA with NAA or IAA combinations were used and the highest percentage of response 98.33% was recorded in MS with 1.0 mg/l BA+0.1 mg/l NAA. The explants produced only axillary shoots but no roots. Another experiment was conducted with different concentration and combination of Kn with NAA or IAA on auxillary shoot proliferation from *in vitro* grown nodal explants of *S. rebaudiana*. From the results it was observed that the highest percentage of response 65.93% was recorded with 1.0 mg/l Kn + 0.1 mg/l NAA. Number of shoots per culture was also recorded highest in the same combination of Kn + NAA.

Different concentration of BA singly or in combination with NAA, IAA or IBA for induction of adventitious shoots from *in vitro* grown internode explants in *S. rebaudiana* were studied and the highest percentage of explants showing shoot proliferation was recorded 98.33% with 1.0 mg/l BA+0.1 mg/l NAA. Similarly different concentration of Kn singly or in combination with NAA, IAA or IBA on induction of adventitious shoots from *in vitro* grown internode explants in *S. rebaudiana*, and Kn with NAA combination showed

best results and the highest percentage of response 60.52% was recorded 1.0 mg/l Kn + 0.2 mg/l NAA and number of shoots per culture was also recorded highest in the same combination of Kn + NAA. For induction of adventitious shoots from *in vitro* grown leaf explants in *S. rebaudiana* BA singly or in combination with NAA, IAA or IBA were used and the combination of 0.5 mg/l BA+0.1 mg/l NAA was found to be the best phytohormone combination. Different concentration of Kn singly or in combination with NAA, IAA or IBA for induction of adventitious shoots from *in vitro* grown leaf explants in *S. rebaudiana* was also studied. The highest percentage of response 60.00% was recorded with 1.0 mg/l Kn + 0.2 mg/l NAA followed by 50.02 with 1.0 mg/l Kn+0.1 mg/l NAA.

Superiority of BA over other cytokinins in producing *in vitro* shoots has also been confirmed in other plants like *Rosmarinus officinalis* (Misra and Chaturavedi, 1984), *Arachis hypogaea* (Mhatre *et al.*, 1985), *Camellia sinensis* (Arulpragasam and Latif, 1986), *Atropa belladonna* (Benjamin *et al.*, 1987) and *Adhatoda vasica* (Amin *et al.*, 1997). Wareing and Phillips (1981) showed that synthetic cytokinin, such a BA, was more active than naturally occurring cytokinin in shoot proliferation. In case of combination of BA with NAA and IAA there is in agreement with the results of *Camellia sinensis* (Phukan and Mitra, 1984); *Capsicum annuum* (Agarwal *et al.*, 1988); *Eucalyptus globosus* (Islam *et al.*, 1994) and other plant species. It was also reported that many plant species like *Piper nigrum* (Geetha *et al.*, 1990); *Coffea arabica* (Raghuramulu *et al.*, 1989); *Chrysanthemum morifolium* (Bhattacharya *et al.*, 1990) and *Dianthus caryophyllus* (Hoque *et al.*, 1996) preferred BA+NAA combination. It was reported that BA-IAA combinations produced shoot buds from *Flaveria trinervia* (Sudarshana and Shanthamma, 1991) and *Ladebouria hyacinthiana* (Turakhia and Kulkarni, 1988) leaves. Barnabas and David (1988) also reported that Kn-NAA combination produced shoots from the leaves of *Solanum surattense*. Barnabas and David (1988) also reported that Kn-NAA

combination produced shoots from the leaves of *Solanum surattense.* The results of the present study are in agreement with the observation of *Solanum sisymbrifolium* and *S. nigrum* (Ara *et al.,* 1993), *S. khasianum* (Baranabas *et al.,* 1989) *Azadiracta indica* (Ramesh and Padhya, 1988; Ramaswamy, 1993), *Pogestemon patchouli* (Misra, 1995), *Rauvolfia serpentina* (Roja *et al.,* 1987) and *Herpestis monnieria* (Sarker and Datta, 1988).

Induction of adventitious roots at the base of the microcuttings prepared from *in viro* grown shoots are indispensable steps to establish tissue culture derived plantlets on the soil. For the rooting purpose IBA, NAA and IAA were used to induce roots on shoots of *S. rebaudiana.* Microcuttings prepared from *in vtiro* proliferated shoots with 3-5 cm length were cultured on $MMS_1$ supplemented meidum with 0.1, 0.2, 0.5 and 1.0 mg/l of IBA, NAA and IAA. Auxin free $MMS_1$ was tested as control. Percentage of root induction and number of roots per shoot cutting were highly influenced by concentration and type of auxins used. No rooting was obtained on auxin-omitted medium. The highest percentage of response 97.66% was recorded in $MMS_1$ with 0.2 mg/l IBA followed by 95.13% with 0.2 mg/l NAA. *In vitro* regenerated plantlets that had been transferred into the soil and 65% of them could tolerate transplantation shock and survive under *ex vitro* environment. Rest of the transplants could not survive either due to desiccation or microbial overgrowth. Damping off and necrosis of the transplants were also observed during acclimatization in *ex vitro* condition of *Eucalyptus tereticomis* (Gill *et al.,* 1993), *Solanum nigram* (Ara *et al.,* 1993), *Rauvolfia serpentina* (Ilahi, 1993) and *Rosa damascena* (Kumar *et al.,* 1995). Being the delicate nature of *in vitro* regenerated plantlets, special arrangement such as controlled green house conditions, use of soil free potting mix like perlite, vermiculite, peat plugs and application of fungicides are to be needed for easy and successful acclimatization of the plantlets (Debergth and Read, 1990).

In the experiment for rooting, the findings are in agreement with those observed in *Chrysanthemum morifolium* (Hoque *et al.*, 1995; Khan *et al.*, 1994), *Solanum tribobatum* (Arockiasamyu *et al.*, 2002) *Carnation* (Radojevic *et la.*, 1990; Hoque *et al.*, 1996), *Cephaelis ipecacuanha* (Jha and Jha, 1989), *Plantago ovata* (Wakhlu and Barna, 1989),*Rheum emodi* (Lal and Ahuja, 1989), *Ruscus hypophyllum* (Jha and Sen, 1985), *Sesbania acculeata* (Bensal and Pandey, 1993), *Pigeon pea* (Sivaprakash *et al.*, 1994), *Vitex negundo* (Thiruvengadam and Jayabalan 2000), *Peganum harmala* (Raman and Jaiwal 2000) and *Psoralea corylifolia* (Jeyakumar and Jayabalan, 2002) as well as many other plant species. Basal callusing was major problem in the emergence of roots, an effect similar to that observed in other plant species (Amin *et al.*, 1997). Stevia is a perennial herb belonging to the Aster family, which is indigenous to the northern regions of South America, Where it is still found growing wild in the highlands of the Amambay and Iguacu distriets (a border area between Brazil and Paraguay). However the plant is now commercially grown in many parts of Brazil, Paraguay, Uruguay, Central America, Isreal, Thailand and China. Through the present investigation techniques for *in vitro* establishment of shoot cultures and further multiplication of shoots and rooting of the shoots have been standardized. Information obtained from different steps of the present study would be of use for initiating any experiment on tissue culture and *in vitro* manipulation of *S. rebaudiana,* a potential now crop for Bangladesh.

# CHAPTER 5

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