REGENERATION POTENTIAL OF SOME MEDICINAL PLANTS

THESIS

By

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Submitted to

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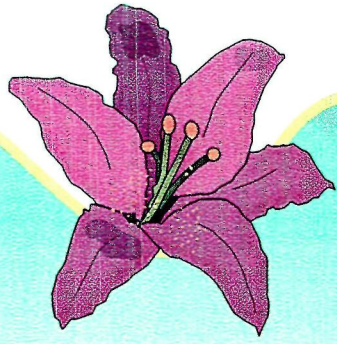

"What can be more clear and sound in explanation, than the love of a parent to his child?"

AFFECTIONATELY
DEDICATED
TO MY
INIMITABLE
PARENTS

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CERTIFICATE – I

This is to certify that the thesis entitled, **"Regeneration potential of some medicinal plants"**, submitted in partial fulfilment of the requirements for the award of the degree of **Master of Science (Agriculture)** in the subject of **Agricultural Biotechnology** of Chaudhary Sarwan Kumar Himachal Pradesh Krishi Vishvavidyalaya, Palampur, is a bonafide research work carried out by **Ms Shveta Sharma (Admission No. A-2005-30-4)** daughter of **Sh. Lekh Raj Sharma** under my supervision and that no part of this thesis has been submitted for any other degree or diploma.

The assistance and help received during the course of this investigation have been fully acknowledged.

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Place : Palampur
Dated: 24/7/07

CERTIFICATE- II

This is to certify that the thesis entitled, **"Regeneration potential of some medicinal plants"**, submitted by **Ms Shveta Sharma (Admission No. A-2005-30-4)** daughter of **Sh. Lekh Raj Sharma** to the CSK Himachal Pradesh Krishi Vishvavidyalaya, Palampur in partial fulfilment of the requirements for the degree of **Master of Science (Agriculture)** in the subject of **Agricultural Biotechnology** has been approved by the Advisory Committee after an oral examination of the student in collaboration with an External Examiner.

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"Aerodynamically the bumblebee shouldn't be able to fly, but the bumblebee doesn't know that, so it goes on flying anyway"

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"Your WORD is a Lamp to guide me and a LIGHT for my path. Your PLAN, O'LORD, I cannot see but ALL IS WELL, that's DONE by THEE".

Place : Palampur
Date : 24th July, 2007.

Shveta Sharma
(Shveta Sharma)

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List of abbreviations

%	Per cent
^{0}C	Degree Celsius
2, 4-D	2,4-dichlorophenoxy acetic acid
BAP	6-Benzyl aminopurine
e.g.	example gratia (for example)
et al.	et alii (and others)
FAA	Formalin Acetic acid Ethyl alcohol
H	Hour (s)
HCl	Hydrochloric acid
i.e.	idest (that is)
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
µM	Micromolar
mg	Milligram
mg/l	Milligram(s) per litre
min	Minute (s)
ml	Milliliter (s)
MS	Murashige and Skoog (1962) medium
NAA	Naphthalene acetic acid
pp.	Pages
Sec	Seconds
PVP	Polyvinylpyrrolidone
PVPP	Polyvinylpolypyrrolidone
STS	Silver thiosulphate
TDZ	Thidiazuron
Kn	Kinetin
TBA	tertiary butyl alcohol
UV	Ultra-violet
V/v	Volume by volume
Viz.	videlicet (namely)
W/v	Weight by volume

Introduction

Chapter- I

INTRODUCTION

Medicinal plants contain compounds of therapeutic value and have been used virtually in all cultures as a source of medicine since time immemorial. Until the development of chemistry and, particularly of the synthesis of organic compounds in the 19th century, medicinal plants and herbs were the sole source of active principles capable of curing a large number of ailments. The World Health Organization estimates that up to 80 per cent of people still rely on herbal remedies for their health care. The natural products are non-necrotic, have no side effects and are easily available at affordable prices and sometimes the only source of health care available to the poor.

Medicinal plants comprise approximately 8000 species and account for around 50 per cent of higher plant species of India. India has a rich biodiversity, but most of the medicinal plants marketed for use in the Indian system of medicine are largely extracted from natural resources. This has greatly contributed towards the alarming depletion of their natural reserves and many of the plant species are in fact, getting endangered and some are facing the threat of extinction. The need of the hour is to develop *ex situ* propagation methods for these plant species and for this purpose, tissue culture methods are best employed for mass multiplication and conservation of elite germplasm. Micropropagated plants can be used to supplement the natural stock of plants in wild as well as to provide a ready supply to the pharmaceutical industries. *Arnebia euchroma* (Royle) Johnston and *Stevia rebaudiana* Bertoni, two important medicinal plants were included in the scope of present study.

***Arnebia euchroma* (Royle) Johnston (Boraginaceae):**

Commonly called as 'Ratanjot'. It is a perennial herbaceous plant, 20-50 cm in height, growing in Himalayas between an altitude range of 3,000-4,200 m above msl. Chromosome number is 2n = 14. Leaves oblong, flowers purplish, white to purple or brownish and in compound cymes. Plant has a thick purple root, is rich in naphthoquinone derivatives such as shikonin, acetylshikonin, ß, ß-dimethyl-acrylshikonin, deoxyshikonin, isovalerylshikonin, isobuthylshikonin (Tabata *et al.*, 1974). These naphthoquinones have antibacterial, anticancer and anti-immunodeficiency activity (Fukui *et al.*, 1999). Shikonin and its derivatives have been used since ancient times as dyes for silk and food products and even today they are used in the dyeing, food and cosmetics industries (Tabata and Fujita, 1985). *A. euchroma* also shows potent anti-HIV activity (Kashiwada *et al.*, 1995). Also, arnebin 1 and arnebin 3, obtained from it, possess anticancerous properties (Harborne and Baxter, 1996).

Although, shikonin and its derivatives are known to be present in the roots of *Alkana tinctoria, Arnebia nobilis* and *Lithospermum erythrorhizon* but *A. euchroma* is known to have much higher pigment contents and is, therefore, regarded as the best source of shikonin-related compounds for industrial applications (Ge *et al.*, 2003).

***Stevia rebaudiana* Bertoni (Asteraceae):**

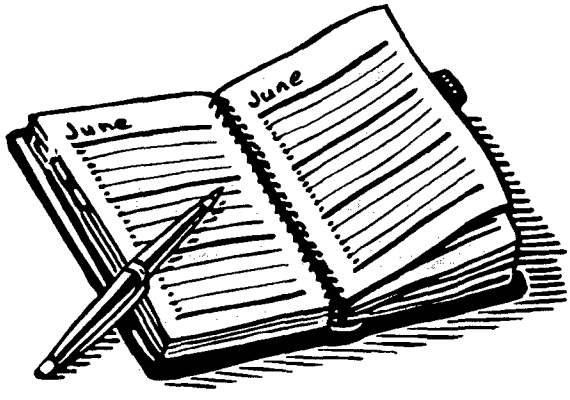
Commonly known as honey grass or sweet plant, sweet leaf, perennial shrub growing to 65-75 cm with alternate leaf arrangement on semi-woody, weak stems; small white flowers arranged in indeterminate heads, self incompatible; seeds small and dispersed by wind through a hairy pappus. Chromosome number is 2n = 22. The glycosides are estimated to be 100-300 times sweeter than sucrose. Eight types of steviol glycosides in leaves have been identified: stevioside, rebaudioside A, rebaudioside B,

rebaudioside C, rebaudioside D, rebaudioside E, steviolbioside and dulcoside A. Stevioside has the largest share of all on dry weight basis (3-10%). It has been used as an intensive non-energetic sweetener in many countries of South America and Asia. Major multinational food companies like Coca Cola and Beatrice foods, convinced of its safety, use *S. rebaudiana* extracts to sweeten foods for sale in Japan, Brazil and other countries where it is approved. It is now been commercially grown in Brazil, Paraguay, Uruguay, Central America, the United States, Israel, Thailand and China.

In *A. euchroma*, there are only a few reports on *in vitro* regeneration such as formation of adventitious buds from callus and leaves (Ji and Wang, 2001), induction of shoot organogenesis on cotyledon and hypocotyl explants (Jiang *et al.*, 2005) and simultaneous organogenesis and somatic embryogenesis (Manjkhola *et al.*, 2005). In *S. rebaudiana*, *in vitro* regeneration is reported from leaves (Yang and Chang, 1979), stem tips (Tamura *et* al., 1984), cell suspension cultures (Ferreira and Handro, 1988a), young leaves (Ferreira and Handro, 1988b), callus (Park and Kim, 2003), shoot apex, nodes and leaves (Sivaram and Mukundan, 2003). Somatic embryogenesis was achieved from leaf explants (Bespalhok *et al.*, 1993) and floret explants (Bespalhok and Hattori, 1997). However, the information available in most of these studies is fragmentary.

Keeping in view the importance of these two medicinal plant species and need for mass multiplication, present studies were carried out with following objectives:

1. Raising of aseptic shoot cultures.
2. Standardization of parameters for direct or indirect regeneration using stem and leaf segments.
3. Attempt to develop regeneration protocol.



Review of Literature

Chapter- II

REVIEW OF LITERATURE

The pharmaceutical industries are totally dependent on medicinal plants for secondary products. Besides providing food and raw materials, the plants are also source of many modern medicines. The demand for plant based products is, therefore ever increasing and to meet the need, the plants are being exploited ruthlessly from their natural habitats. With the result, many economically important plants are threatened.

In order to save this precious wealth, there is need for conservation of planting material employing tissue culture techniques. Two species of important medicinal plants viz. *Arnebia euchroma* (Royle) Johnst and *Stevia rebaudiana* Bertoni were included in the present study and information regarding their micropropagation and regeneration are reviewed in the following pages:

2.1 *Arnebia euchroma* (Royle) Johnston

Due to overexploitation of natural resources, *Arnebia euchroma* has been described as the most threatened medicinal plant species (Kala, 2005). Therefore, an efficient *in vitro* propagation and regeneration system is required for the production and conservation of planting material. A perusal of literature reveals that there are a few reports on micropropagation and regeneration of this medicinally important plant species.

2.1.1 Plant Regeneration

There are three reports on plant regeneration in *A.* euchroma (Table 1). Ji and Wang (2001) used callus and leaves for regeneration purpose. Young buds on modified MS medium supplemented with NAA (0.4 mg/l), 2, 4-D (0.2 mg/l) and Kn (1.0 mg/l) induced callus formation. Adventitious buds were produced after 3 months from callus

Table 1: Regeneration and somatic embryogenesis in *A. euchroma*

S. No.	Explant	Medium	PGRs/Additives	Response	Remarks	Authors
1.	Young leaves	LS	NAA (0.4 mg/l) + Kn (1.5 mg/l)	Adventitous buds	Direct organogenesis	Ji and Wang, 2001
	Callus derived from young buds	Modified MS	NAA (0.4 mg/l) + Kn (2.5 mg/l) or BAP (1.2 mg/l)	Adventitious buds	Indirect organogenesis	
2.	Cotyledons and Hypocotyls	LS	TDZ (4.5 µM)	8.6 shoots per cotyledon explant, 5000 plants produced within 6 months	Problem of browning of shoots overcome by PVP (33.3 µM)	Jiang et al. , 2005
3.	Leaf derived callus	MS	IBA (1.0 µM) + BAP (2.5 µM)	Indirect organogenesis	Synthetic seeds also produced using somatic embryos; germination frequency on MS medium was 66.6%	Manjkhola et al., 2005
			IBA (2.0 µM)	Rooting		
			IBA (2.5 µM) + BAP (2.5 µM)	Somatic embryogenesis		

cultured on LS medium supplemented with NAA (0.4 mg/l) and Kn (2.5 mg/l) or BAP (1.2 mg/l). Kn was found to be better than BAP. However, direct formation of adventitious buds was evident from young leaves cultured on modified MS medium containing NAA (0.4 mg/l) and Kn (1.5 mg/l). The chromosome number in root tips was 2n = 14 indicating, thereby the stability of regenerated plantlets.

There is only one report on shoot organogenesis using cotyledons and hypocotyls as explants (Jiang *et al.*, 2005). Thidiazuron (4.5 μM) resulted in 8.6 shoots per cotyledon explant on LS medium after 12 days of culture. Other PGRs like BAP, Kn, NAA were found to be ineffective. Browning of shoots was a common problem which could be eliminated by addition of Polyvinylpyrrolidone (PVP; 33.3 μM). For root induction, IBA (1.0 mg/l) was required. Within a period of 6 months, 5000 plants were produced.

Manjkhola *et al.* (2005) reported simultaneous indirect organogenesis and somatic embryogenesis from leaf derived callus. Organogenesis was obtained on MS medium added with IBA (1.0 μM) + BAP (2.5 μM) and somatic embryogenesis occurred on medium supplemented with IBA (2.5 μM) and BAP (2.5 μM). Rooting of shoots was achieved in IBA (2.0 μM). Young somatic embryos were used for the production of synthetic seeds which germinated (66.6%) in MS medium.

2.1.2 Callus and Cell Suspension culture

The work on tissue culture of *A. euchroma* started in early nineties. The first report of callus and cell suspension culture was that of Dong *et al.* (1993). They observed that during the course of cell growth, the pH of the medium changed and then ceased after 12 days of culture. In the liquid medium, the maximum shikonin contents were observed by day 25. The cell suspensions were up scaled in 10 litre bioreactor.

Chen *et al.* (1994) described progressive scale-up culture system from 250ml shake flask to 9 litre and 25 litre airlift bioreactors. Production of shikonin derivatives was found to be inversely proportional to cell growth. Xiao *et al.* (2004) reviewed the work on callus and cell suspension culture and up scaling using bioreactors. In India, there is no report of cell culture.

2.2 *Stevia rebaudiana* Bertoni

The sweetening properties of *S. rebaudiana* are due to the presence of diterpene glycosides. Seed raised progenies are heterogeneous and variability with respect to constituents responsible for sweetening is observed (Tamura *et al.*, 1984; Nakamura and Tamura, 1985). In addition, *S. rebaudiana* seeds show low germination percentage (Felippe *et al.*, 1971; Felippe and Lucas, 1971). Vegetative propagation has also limitations as it is difficult to obtain large population from single plant (Sakaguchi and Kan, 1982). Therefore, for rapid mass multiplication tissue culture is the best alternative.

There are a few reports on micropropagation and regeneration in *S. rebaudiana* and these are described as follows:

2.2.1 Plant regeneration

The information on plant regeneration in *S. rebaudiana* is summarized in table 2. The earliest report on plant regeneration is of direct shoot regeneration using leaflets of 12-16 day old seedlings (Yang and Chang, 1979). Multiple shoots were formed on modified MS medium supplemented with 2.0-10.0 mg/l BAP. Rooting of regenerated shoots occurred on PGR free MS medium or on moistened vermiculite and subsequently these were transferred to soil.

Table 2: Regeneration and somatic embryogenesis in *S. rebaudiana*

S. No.	Explant	Medium	PGRs/Additives	Response	Remarks	Authors
1.	Leaflets of 12-16 day old seedlings	MS	BAP (2.0-10.0 mg/l)	Multiple shoots	–	Yang and Chang, 1979
			–	Rooting		
2.	Stem tips with few leaf primordia	MS	Kn (10.0 mg/l)	Adventitious buds from leaf margins	–	Tamura *et al.*, 1984
			NAA (0.1mg/l)	Rooting of micro-shoots		
3.	Callus formed from cell suspension cultures	LS (Macro and microelements); vitamins (Nitsch, 1969)	Kn (2.0 mg/l) + NAA (0.02 mg/l)	Adventitious buds	Callus was derived from foliar discs	Ferrerira and Handro, 1988a
			–	Shoots and root development		
4.	Young leaves	Not mentioned	BAP (2.0 mg/l)	Shoot regeneration	Cultured in light	Ferrerira and Handro, 1988b
		Not mentioned	BAP (2.0 mg/l) + NAA (2.0 mg/l)	Shoot regeneration	Cultured in dark	
		Not mentioned	BAP (0.1 mg/l)	Rooting	–	
5.	Shoot apex, nodes, leaves	MS	BAP (8.87 µM) + IAA (5.71 µM)	Shoot induction	Callus formed on BAP (8.87 µM) + IBA (9.80 µM) was found to have highest sweetener content	Sivaram and Mukundan, 2003
6.	Leaves, seeds, hypocotyls, cotyledons	MS	NAA (1.0 mg/l) + Kn (3.0 mg/l)	Calluls, adventitious buds	Regeneration from seed derived callus was 23.8%	Park and Kim, 2003
7.	Leaf Explants	MS + Sucrose (12%)	BAP (1.0 µM)+ 2, 4-D (10.0 or 25.0 µM)	Somatic embryos	Embryos did not develop further	Bespalhok *et al.*, 1993
8.	Floret explants	MS	Kn (0-9.29 µM)+ 2, 4-D (9.05 or 18.10 µM)	Indirect embryogenesis	Histological studies confirmed unicellular origin of somatic embryos	Bespalhok and Hattori, 1997

Tamura *et al.* (1984) cultured stem-tips with a few leaf primordia on MS medium supplemented with Kn (10.0 mg/l). Adventitious buds formed on leaf margins gave rise to multiple shoots. Rooting was achieved on medium containing NAA (0.1 mg/l) without Kn.

Cell suspension cultures have also been used for regeneration (Ferreira and Handro, 1988a). Cultures derived from actively growing calli (leaf derived) were mainly diploid, whereas those from senescent calli had a wide variation in chromosome number. Cell suspensions were maintained for 3 years in liquid LS medium containing BAP (0.5 mg/l) and 2, 4-D (1.0 mg/l). Isolated cells and cell aggregates formed callus when plated on agar gelled LS medium containing BAP and 2, 4-D (0.5 mg/l each). These calli (diploid ones) formed adventitious buds when transferred to medium supplemented with Kn (2.0 mg/l) and NAA (0.02 mg/l). For further shoot development and rooting, LS medium without PGRs was used. In addition to diploids, some tetraploids and aneuploids regenerated plants were noticed.

Young leaves from adult plants were cultured in light on BAP (2.0 mg/l) or in dark on BAP (2.0 mg/l) + NAA (2.0 mg/l) (Ferreira and Handro, 1988b). The regenerated shoots were transferred to BAP (0.1 mg/l) and with continuous sub-culturing shoots ready for rooting were obtained. BAP (0.1 mg/l) favoured root induction. The plants thus formed showed uniform chromosome number of 2n= 22. Colchicine treated plants with altered chromosome number were also obtained.

Sivaram and Mukundan (2002) presented the cost involved in the micropropagation and hardening plantlets in greenhouse in India. Subsequently in 2003, they obtained shoot regeneration using shoot apex, nodes and leaves as explants. This paper advocates the use of MS medium containing BAP and IAA for shoot induction and

half strength MS medium with IBA for rooting of shoots. The explants were cultured on MS medium containing BAP (8.87 μM) and IAA (5.71 μM). Callus was formed on agar-gelled MS medium supplemented with BAP (8.87 μM) and IBA (9.80 μM). The callus thus formed was found to have the highest sweetener content and indicated that even disorganized callus tissues could be a potential source of the sweetening compounds of *S. rebaudiana.*

Park and Kim (2003) studied the factors affecting callus induction and plant regeneration using different explants such as leaves, seeds, hypocotyls and cotyledons. Frequency of callus formation was higher in young leaf explants than the older ones and the frequency was also higher in the seeds than hypocotyls and cotyledons on MS medium supplemented with 2, 4-D (1.0 mg/l). Per cent regeneration response from seed derived embryogenic callus was 23.8 on MS + NAA (1.0 mg/l) and Kn (3.0 mg/l).

2.2.2 Somatic embryogenesis

Bespalhok *et al.* (1993) found that somatic embryos were formed directly from leaf explants cultured on MS medium supplemented with 2, 4-D (10.0 or 25.0 μM) and BAP (1.0 μM) and a high concentration of sucrose (12%). These embryos, however, did not mature to develop shoots when cultured on MS medium without PGRs and low concentration of sucrose (3%) but only roots were formed.

In another report on somatic embryogenesis Bespalhok and Hattori (1997) used floret explants. These were cultured on MS + 2, 4-D (9.05, 18.10 μM) and Kn (0-9.29 μM). They showed that maximum embryogenic callus was formed on 2, 4-D (9.05 μM) without Kn. 2, 4-D (9.05 μM) either alone or along with 2.32 μM Kn gave best results. On the basis of histological studies, unicellular origin for the somatic embryos was proposed.

2.2.3 Shoot multiplication

Shoot multiplication in *S. rebaudiana* was reported by a number of workers (Table 3). First report of shoot multiplication is that by Yang (1981) using cotyledon explants. Miyagawa *et al.* (1986) developed a method to form plantlets from shoot primordia. Shoot tips were used to induce shoot primordia on Gamborg B5 medium containing BAP and NAA which subsequently developed into plantlets when cultured on B5 medium containing 0.02 mg/l BAP and sucrose (2%).

Bespalhok *et al.* (1992) studied the factors influencing the micropropagation of axillary shoots. Nodal segments 2.0 cm in length obtained from adult plants were cultured on MS medium containing 6 levels of ammonium nitrate (NH_4NO_3), De Fossard vitamins, BAP (11.0 μM), sucrose (3%) and agar (1%) for shoot multiplication. When the concentration of NH_4NO_3 was lowered from standard 20.60 mM to 5.15 mM in the multiplication medium, there was increase in the number of shoots produced per nodal segment to an average of 10.90. An increase in the number of roots / shoot was obtained when MS salt level was decreased in the rooting medium by half along with 10.0 μM of NAA.

Kornilova and Kalashnikova (1996) recommended MS medium without growth regulators for clonal propagation of *S. rebaudiana*, using nodal segments with 2 axillary buds. IAA (0.5 mg/l) was used for root induction. On the contrary, Constantinovici and Cachita (1997) found that shoot apices are better explants than nodal stem segments for *in vitro* multiplication of *S. rebaudiana*. Also BAP was proved to be more effective than Kn in multiplication medium. Rooting took place on either PGR free medium or medium containing Kn.

Table 3: Shoot multiplication in *S. rebaudiana*

S. No.	Explant	Medium	PGRs/Additives	Response	Remarks	Authors
1.	Shoot tips	Gamborg's B5	BAP (0.02 mg/l) + Sucrose (2%)	Formation of plantlets	–	Miyagawa *et al.*, 1986
2.	Nodal segments	MS containing 6 levels of NH_4NO_3, De Fossard vitamins	BAP (11.0 µM)	Multiple shoots	At 5.15mM NH_4NO_3, increased no. of shoots per nodal segment to an average of 10.90	Bespalhok *et al.*, 1992
		Half strength MS	NAA (10.0 µM)	Rooting	Increase in number of root/shoot	
3.	Nodal segments with 2 axillary buds	MS	–	Multiple shots	–	Kornilova and Kalashnikova, 1996
			IAA (0.5mg/l)	Rooting		
4.	Shoot apices	Not mentioned	BAP	Shoot multiplication	–	Constantinovici and Cachita, 1997
			Kn or No PGR	Rooting		
5.	Nodal segments of 6 week old seedlings	MS with 50% macroelements	NAA (0.1ppm)	Shoot multiplication	Increase in stevioside content of leaves in Humiforte and Aminol and highest biomass yields in Melatran	Acuna *et al.*, 1997
			IAA (5%)	Rooting	Nodal segments given a dip	
6.	Nodal segments obtained from seedlings	MS	IBA (5.0 mg/l) + NAA (2.0 mg/l)	Multiple shorts	–	Maharik and El, 2003
			BAP (4.0 mg/l) + NAA (2.0 mg/l)	Callusing	Poor shoot multplication	
		Half strength MS	IBA (concentration not mentioned), Activated charcoal (0.1%)	Rooting	Activated charcoal improved root development	
7.	Axillary shoot buds, terminal shoot buds and leaf segments	MS	BAP (1.0 mg/l)+ Kn (0.05 mg/l)	Multiple shoots	Rooting more than 90%	Smitha *et al.*, 2005
			IBA(1.5 mg/l)	Rooting		
			BAP (1.0 mg/l) + NAA (0.1 mg/l)	Regeneration from leaf explants		

Acuna *et al.* (1997) cultured nodal segments of 6-week old seedlings on MS medium with 50 per cent macroelements and 0.1 ppm NAA. Addition of humiforte (synthetic amino acids N, P, K and trace elements) in combination with Aminol (amino acids and N) favoured increase in stevioside content in leaves but Melatran (lactic acid and anthranilic acid) gave the highest biomass yields. Nodal segments were dipped in IAA (5%) solution for root induction.

Effects of IAA and BAP in MS medium were studied on propagation of *S. rebaudiana* from meristems (Zbughin *et al.*, 2002). They presented mathematical model on effect of different PGRs on morphological characteristics like number of leaves, height of stemlets, number of axillary buds, and number of basal rootlets.

Effects of two cytokinins: Kn and BAP was studied among 4 different genotypes (Morini *et al.*, 2003). BAP was found to be associated with susceptibility to tissue vitrification, very small leaves, thin and etiolated stem apical portions. Whereas with Kn, symptoms were less pronounced. Rooting response was evaluated by IBA at 0.5 and 0.1 mg/l concentration. Among all the genotypes, shoot proliferation ranged from about 1: 4 to 1: 9 and rooting from 65 to 92 per cent. Hardening of plantlets posed severe problems. In general response was genotype specific.

Maharik and El (2003) cultured nodal segments obtained from seedlings on MS medium supplemented with various concentrations of BAP, NAA and IBA. Highest number of shoots was obtained from medium containing IBA (5.0 mg/l) and NAA (2.0 mg/l). When BAP was combined with IAA, callusing occurred. When the concentration of BAP and NAA was reduced to 4.0 mg/l and 2.0 mg/l respectively callusing took place with poor shoot multiplication. Rooting was induced on half strength MS medium supplemented with IBA + sucrose (20%) + activated charcoal (0.1%) and agar (0.7%). Addition of activated charcoal, to the medium, improved root development.

MS medium supplemented with BAP (1.0 mg/l) and Kn (0.05 mg/l) proved to be stimulatory for *S. rebaudiana* mass multiplication from axillary shoot buds, terminal shoot buds and leaf segments (Smitha *et al.,* 2005). More than 90 per cent rooting was achieved when shoots were cultured on modified MS medium supplemented with 1.5 mg/l of IBA. Direct shoot regeneration was also evident from leaf explants on modified MS medium supplemented with 1.0 mg/l BAP and 0.1 mg/l NAA. The regenerated plants were true to type to mother plant with respect to sweetening taste as per organoleptic testing.

2.2.4 Upscaling of shoot multiplication in bioreactor

Another procedure for mass propagation of *S. rebaudiana* has been described (Table 4). Akita *et al.* (1994) inoculated isolated shoot primordia to obtain clusters of shoots. These were subsequently grown in a 500 l bioreactor for shoot multiplication in modified MS (inorganic salts reduced to half) + sucrose (1%). 64.6 kg of shoots were propagated from 460 g of isolated shoot primordia.

Nepovim and Vanek (1998) used 300 ml of medium to upscale shoot cultures in a bioreactor assembled from an agitator, agitated by a magnetic mixer, and a semi permeable tubing system providing aeration (Table 4). Nodal segments were used for raising multiple shoot culture in MS medium containing half strength macroelements, sucrose (1%) and NAA (0.01 mg/l). Shoots were sub-cultured on MS medium supplemented with BAP (2.1 mg/l). The shoots thus produced were inoculated (2.0 g; fresh weight) in 300 ml liquid MS medium devoid of PGRs. After 4 weeks of cultivation, 50-60 shoots were obtained.

Table 4: Upscaling of shoot multiplication of *S. rebaudiana* in bioreactor

S. No.	Explant	Medium	PGRs/Additives	Response	Remarks	Authors
1.	Shoot primordia	Modified MS (inorganic salts reduced to half) + sucrose (1%)	–	Multiple shoots	64.6 kg of shoots propagaed from 460 g of isolated shoot primordia in 500 l bioreactor	Akita *et al.*, 1994
2.	Nodal segments	MS liquid	–	Multiple shoots	50-60 shoots formed in assembled bioreactor (300 ml medium used)	Nepovim and Vanek, 1998

2.2.5 Callus culture

Handro *et al.* (1997) found vigorous callus growth from leaf discs and 1.0 cm long internodal stem segments cultured on either IAA or 2, 4-D and BAP or Kn containing medium. Lee *et al.* (1982) found that optimum concentration for callus induction was BAP 1.0 μM and NAA 0.1 μM. Stevioside content was also analyzed through TLC-FID which was 260 mg per 100 g (dry weight) in the leaf derived callus.

Callus was initiated from leaves on MS medium supplemented with combinations of auxins i.e. IAA, IBA, NAA, 2, 4-D at 1.0 mg/l each and cytokinin i.e. Kn at 0.1, 0.2 or 0.3 mg/l (Das *et al.*, 2005). Different strengths of MS medium were used for initiation and maintenance of callus. Callus was best initiated on half strength MS medium supplemented with 2, 4-D (1.0 mg/l) and Kn (0.2 mg/l), whereas for callus maintenance combination of NAA (0.1 mg/l) and BAP (2.0 mg/l) was found to be optimum. Callus on half strength medium was found to have potential to increase stevioside content as estimated by HPTLC.

Although, micropropagation studies have been carried out in both the plant species included in the present studies, yet different response is evident in different reports. Therefore, an attempt was made to study regeneration potential of leaf and stem segments in *A. euchroma* and *S. rebaudiana.*

Materials and Methods

Chapter- III

MATERIALS AND METHODS

3.1 Planting Material and Explants Used

Plants of *Arnebia euchroma* (Royle) Johnston and *Stevia rebaudiana* Bertoni growing at the experimental farm of Institute of Himalayan Bioresource Technology, Palampur were used as source of explants.

3.1.1 Explants used:

3.1.1.1 *Arnebia euchroma*:

a) Shoot Multiplication:

i) Rhizome buds from *ex vitro* grown plants

ii) Shoots (1.0-1.3 cm in length from established cultures)

b) Regeneration:

i) Leaves (0.75-1.0 cm^2) from *in vitro* shoot cultures

ii) *In vitro* shoots having 2-6 leaves (0.5-1.0 cm^2)

iii) Callus derived from leaves of *in vitro* shoots

3.1.1.2 *Stevia rebaudiana*:

a) Shoot Multiplication:

i) Nodal segments (1.0-3.0 cm in length)

b) Regeneration:

i) Seeds

ii) Seedlings (1.0-1.5 cm long; 2 week old)

iii) Shoots from shoot cultures having 3-4 leaves (1.0 – 1.5 cm^2)

iv) Fully expanded third leaf (2.0-3.0 cm^2) from top of *ex vitro* grown plants

v) Leaves (1.0-1.3 cm^2) from *in vitro* shoot cultures

vi) Callus derived from both *ex-vitro* as well as *in vitro* leaves

3.2 Glassware Used

Erlenmeyer conical 250 ml flasks (Borosil, India), autoclavable petriplates (90 mm diameter; HiMedia Laboratories Pvt. Limited, India) were used for shoot multiplication and regeneration studies, respectively unless otherwise specified.

3.3 Culture Medium

Murashige and Skoog (1962) medium (MS; Appendix 1) was invariably used. Stock solutions (Appendix 2) of major salts, minor salts and vitamins were prepared in double distilled water and stored in refrigerator at 4 ^{o}C. Agar (0.8%; w/v) and sucrose (3%; w/v) were added invariably to the medium unless otherwise specified. The pH of the media was adjusted to 5.6-5.8 with either 0.1N HCl or 0.1N KOH before autoclaving. The media were autoclaved for 20 minutes at 1.1 kg/cm^2 pressure and 121 ^{0}C temperature.

3.3.1 Plant Growth Regulators (PGRs) and other additives

3.3.1.1 *Arnebia euchroma:*

IBA (5.0, 10.0 µM), NAA (5.0 µM), Picloram (0.1, 0.5, 1.0 µM), BAP (0.01, 0.05, 0.1, 0.5, 1.0, 2.5, 3.0, 5.0, 10.0 µM), Kinetin (0.01, 0.05, 0.1, 0.5, 1.0, 2.5, 3.0, 5.0, 10.0 µM), TDZ (1.0, 2.5, 5.0, 10.0, 15.0, 20.0 µM).

In one set of regeneration experiment, *silver nitrate (15.0, 35.0 and 50.0 mg/l) was also used.

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* Filter sterilized and added to the respective media after autoclaving unless otherwise specified.

3.3.1.1 (a) Adsorbents

Polyvinylpyrrolidone (PVP), Polyvinylpolypyrrolidone (PVPP), activated charcoal and ascorbic acid at 100 mg/l concentration each.

3.3.1.1 (b) Control of contamination

In order to control bacterial contamination in the shoot cultures of *A. euchroma*, three types of antibiotics at varying concentrations were separately added to the medium. These were *kanamycin (50.0, 100.0 and 250.0 mg/l), *streptomycin sulphate (50.0, 100.0 and 250.0 mg/l), *cefotaxime (50.0, 100.0 and 250.0 mg/l).

3.3.1.2 *S. rebaudiana*:

*IAA (5.77 µM), NAA (1.0, 2.5, 5.0 µM), 2, 4-D (2.5, 5.0, 10.0, 15.0, 25.0 µM), BAP (0.5, 1.0, 2.5, 5.0, 8.87 µM), Kn (1.0, 2.5, 5.0 µM).

*Silver thiosulphate (STS; 2.5, 5.0 µM; Appendix 3), silver nitrate (15.0, 35.0 mg/l) were also used.

In one set of regeneration experiment, increased sucrose concentrations (6%, 12%; w/v) in the medium along with 1.0 µM BAP and 10.0 and 25.0 µM 2, 4-D were used.

In both the species, the medium without PGRs and additives served as control.

3.4 Surface Sterilization

3.4.1 *A. euchroma*:

Rhizome buds were rinsed in running tap water for 10-15 minutes and cleaned with Tween-20 (HiMedia Laboratories Pvt. Limited, India) using sable hairbrush. Then these were treated with (0.03%; w/v) bavistin (BASF, India) and (0.03%; w/v)

* Filter sterilized and added to the respective media after autoclaving unless otherwise specified.

streptomycin sulphate (Sigma, India) solution for 10-15 minutes. Thereafter the explants were washed with distilled water. After this step, the explants were taken to Laminar Air Flow Cabinet. These were then dipped in ethanol (70%; v/v), treated for 5-7 minutes with mercuric chloride (0.03%; w/v) containing 1-2 drops of Tween-20 as wetting agent and subsequently washed 3-4 times with autoclaved double distilled water.

3.4.2 *S. rebaudiana*:

Stem segments containing at least 3-4 nodes and seeds were taken and cleaned using sable hairbrush with Tween-20 and (70%; v/v) ethanol. This was followed by treatment with (0.01%; w/v) bavistin and (0.01%; w/v) streptomycin sulphate for 10 minutes. Thereafter these were washed with distilled water. The subsequent steps for surface sterilization of explants were performed in the Laminar Air Flow Cabinet. The explants were given treatment with mercuric chloride (0.02%; w/v) containing 1-2 drops of Tween-20 as wetting agent for 4-5 minutes. Afterwards 3-4 washings were given with autoclaved distilled water.

For leaves, the procedure described is the same as above except for concentration and time duration of ethanol and mercuric chloride solution, which were 50 per cent (v/v) and 0.01 per cent (w/v) respectively for 2-3 minutes.

3.5 Inoculation and Incubation

3.5.1 *A. euchroma:*

(a) Shoot multiplication:

i) Raising of aseptic cultures: The cut ends of the rhizome buds which came in contact with surface sterilant were removed. These rhizome buds were then inoculated vertically on MS medium supplemented with 10.0 μM BAP along with 5.0 μM NAA. These PGRs were selected on the basis of previous

experiments in our laboratory. The medium was also supplemented with bavistin (50.0 mg/l) and streptomycin sulphate (250.0 mg/l) added prior to autoclaving. The cultures were incubated at 25±2 ^{0}C with Photosynthetic Photon Flux Density (PPFD) of 70 µmol $m^{-2}s^{-1}$ supplied from cool white fluorescent tubes and maintained on 16 hours light and 8 hours dark cycle.

ii) Shoot proliferation: Shoots obtained on BAP (10.0 µM) + NAA (5.0 µM) containing medium were maintained by regular sub-culturing at 25-30 days interval. To study the effect of different PGRs on shoot multiplication, shoots with 3-4 leaves obtained on BAP (10.0 µM) + NAA (5.0 µM) were transferred to different concentrations of BAP (2.5, 5.0, 10.0 µM) or Kn (1.0, 2.5, 5.0, 10.0 µM) or TDZ (5.0, 10.0, 15.0, 20.0 µM) and incubated in culture room in light conditions as described above in section 3.5.1(a).

(b) Regeneration studies:

i) Leaves from *in vitro* culture were segmented into three parts i.e. basal, middle and tip. Complete leaves from *in vitro* cultures were also used. In both the cases, leaves were inoculated with their abaxial surface in contact with the medium. Medium employed was MS medium supplemented with picloram (0.1, 0.5, 1.0 µM), TDZ (1.0, 2.5, 5.0 µM), BAP (10.0 µM) + IBA (5.0µM) / NAA (5.0 µM). These cultures were incubated in culture room under dark conditions.

ii) Shoots with 2-3 leaves (0.5-1.0 cm^2) were inoculated vertically on MS medium + TDZ (20.0 µM) supplemented with cefotaxime and PVPP (100.0 mg/l each) in order to check systemic bacterial contamination and phenolic

exudation, respectively from explants in 250 ml flasks. Shoots were cultured for 20, 40 and 60 days on this medium before transferring to PGR free medium in culture room under light conditions.

In another experiment, shoots with 5-6 leaves (0.5-1.0 cm^2) were cultured for 40 days on MS medium containing high concentration of TDZ i.e. 20.0 μM and 100.0 mg/l PVPP and subsequently transferred to lower concentration of TDZ (5.0 μM). Cultures were incubated in culture room in light conditions.

iii) Callus derived from *in vitro* leaves obtained on BAP (10.0 μM) + IBA (5.0 μM) containing medium was inoculated on MS medium supplemented with BAP (0.01, 0.05, 0.1, 0.5, 1.0, 3.0, 5.0, 10.0 μM) + IBA (10.0 μ M), Kn (0.01, 0.05, 0.1, 0.5, 1.0, 3.0, 5.0, 10.0 μM) + IBA (10.0 μM). Callus formed at the base of shoots on MS medium supplemented with TDZ (20.0 μM) containing media was also transferred to TDZ (5.0 μM) or silver nitrate (15.0, 35.0 and 50.0 mg/l) and incubated in culture room in light conditions.

3.5.2 *S. rebaudiana*:

(a) Shoot multiplication:

i) Raising of aseptic cultures: The cut ends which came in contact with surface sterilization agents were removed from the nodal segments containing two nodes. These segments were inoculated on MS medium supplemented with BAP (2.5, 5.0 μM) or STS (2.5, 5.0 μM). Bavistin (50 mg/l) and streptomycin sulphate (250 mg/l) were also added to these media. The cultures were incubated in culture room in light conditions as described in 3.5.1(a).

ii) Shoot proliferation and maintenance of cultures: The cultures on BAP (2.5, 5.0 μM) were screened for contamination for 15-21 days and sub-culturing was done after 15 day interval. Medium containing STS (2.5 μM) was used for maintenance of cultures.

(b) Regeneration studies:

i) *Ex vitro* leaves were segmented into basal, middle and tip portion and were inoculated with their abaxial surface in contact with the medium. MS medium was supplemented with Kn (1.0, 2.5, 5.0 μM) + NAA (1.0, 2.5, 5.0 μM), BAP (0.5, 1.0, 2.5 μM) + 2, 4-D (2.5, 5.0, 10.0 15.0 μM) in factorial combinations. *In vitro* leaves were inoculated as such without being segmented because of their smaller size (1.0-1.3 cm^2). These were inoculated on MS medium supplemented with BAP (1.0 μM) + 2, 4-D (10.0, 25.0 μM) + sucrose (6%, 12%).

Leaves from *in vitro* shoot cultures raised on MS medium supplemented with STS (2.5 μM), were inoculated on MS medium supplemented with silver nitrate (15.0, 35.0 mg/l).

ii) Callus formed from *in vitro* leaf explants on MS medium supplemented with BAP (0.5, 1.0, 2.5 μM) + 2, 4-D (2.5, 5.0, 10.0 15.0 μM) and Kn ((1.0, 2.5, 5.0 μM) + NAA (1.0, 2.5, 5.0 μM) was transferred to PGR free medium.

iii) Nodal (1-1.5 cm long; 2 nodes each) and internodal segments (1-1.5 cm long) were inoculated horizontally on MS medium supplemented with silver nitrate (15.0, 35.0 mg/l).

iv) Seeds were inoculated on PGR free MS medium and were incubated in dark conditions in culture room.

iv) Seedlings (1.0-1.5 cm long; 2 week old) were segmented into 3 parts i.e. shoot, hypocotyl and root portion. These were inoculated on MS medium containing BAP (8.87 μM) + IAA (5.77 μM).

3.6 Histological studies

i) Killing, fixing and dehydration of material: The material was fixed in FAA (Formalin : Acetic Acid : Ethanol 50% :: 1 : 1 : 18) for 1 week. The material was preserved in 70% ethanol until use after which the tissue was dehydrated in the following TBA (t-butyl alcohol) series:

Rect. alcohol (ml)		:	TBA (ml)
a)	30	:	20
b)	50	:	20
c)	50	:	35
d)	45	:	55
e)	25 (Ethanol)	:	75
f)	-	:	100

Water was added to each grade (a-c) to make total volume up to 100 ml. The material was kept in each grade for 2 hours and was kept overnight in sixth grade.

ii) Infiltration: The material was kept in an oven at 60°C and paraffin wax flakes were added after every 15-20 min. The whole process was carried out for a minimum of 4 h. Finally, it was kept in an oven (60°C) overnight.

iii) Block making and section cutting: Blocks were made and sections (12 μm) thick were cut with the help of a rotary microtome (Finsee, ME, USA).

iv) Mounting and stretching: A drop of adhesive (1% gelatin) was applied on a clean slide after which 2-3 drops formalin (4%) were put on the slide and sections placed on it. These were stretched on a hot plate at 50°C and kept overnight at room temperature properly covered in order to avoid dust.

v) Dewaxing and Staining: Slides were passed through each of the following grades for the time specified:

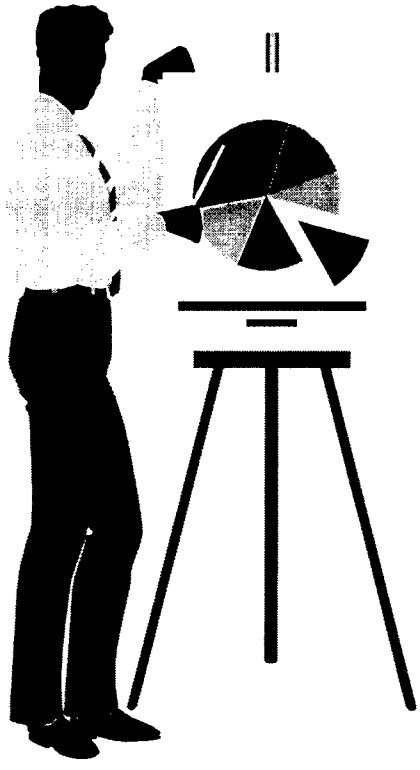
	Grades		Time
a)	Xylene I	:	3 min
b)	Xylene II	:	3 min
c)	Xylene : Absolute alcohol (1:1)	:	75
d)	Absolute alcohol I	:	4 min
e)	Absolute alcohol II	:	4 min
f)	Rectified spirit	:	2 min
g)	70% alcohol	:	2 min
h)	50% alcohol	:	2 min
i)	Safranin	:	4 hours
j)	50% alcohol	:	45 sec
k)	70% alcohol	:	45 sec
l)	90% alcohol	:	45 sec
m)	Rectified spirit	:	45 sec
n)	Absolute alcohol I	:	45 sec

o)	Absolute alcohol II	:	45 sec
p)	Clove oil	:	1 min
o)	Fast green	:	just dip
q)	Clove oil II	:	1 min
r)	Clove oil : Xylene (1:1)	:	1 min
s)	Xylene I	:	2 min
t)	Xylene II	:	5 min

vi) Mounted the slides in D.P.X. mountant (s.d. fine-chem limited)

3.7 Statistical analysis

In each experiment, 3-4 replicates with 6 explants per culture vessel were taken. Each experiment was repeated twice. Their mean and standard deviation were calculated.



Results

RESULTS

4.1 *A. euchroma*

4.1.1 Shoot multiplication

Fig. 1 Bud sprouting after 20-25 days

i) **Raising of aseptic cultures:** Buds sprouted (Fig. 1) after 20-25 days of culture on MS medium fortified with 10.0 µM BAP + 5.0 µM NAA + 50 mg/l bavistin and 250 mg/l streptomycin sulphate. These were then transferred to medium devoid of antifungal and antibiotic agents. It was observed that cultures remained aseptic for 4 weeks, after which these became necrotic due to systemic bacteria.

Therefore, different antibiotic agents viz., kanamycin, streptomycin sulphate and cefotaxime were added to media in order to avoid the re-occurrence of bacteria. Although problem of systemic bacteria could be circumvented at all the concentrations of cefotaxime (used in experiment) but at concentration lower than 100.0 mg/l there was re-occurrence of bacteria after 2-3 subculturings and at higher concentration, cultures perished. On the other hand, on kanamycin or streptomycin sulphate containing medium shoot growth was hampered and problem of bacterial contamination was not lled. Therefore, cefotaxime at 100.0 mg/l concentration, when added to medium wa d to be most effective in eliminating the systemic bacteria without having adverse effect on shoot growth. Another problem in the establishment of shoot cultures was release of phenolics into the medium, which was controlled by the addition of different additives, viz., ascorbic acid, activated charcoal, PVP or PVPP. Of these, PVPP (100.0 mg/l) was proved to be the most suitable in controlling phenolic exudation into the medium.

ii) **Shoot multiplication:** Shoot cultures were maintained on BAP (10.0 µM). Although there was about 8 fold increase in shoot number after 30 days but these became vitrified after regular sub-culturing.

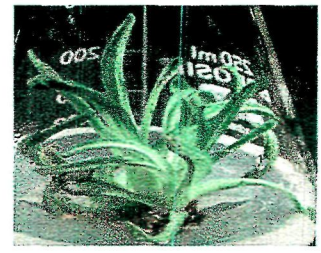


Fig. 2 Shoot proliferation on Kn 5 µM

Even lower and higher concentrations of BAP could not alleviate vitrification. Kn at different concentrations was used (Table 5) and it was observed that there was about 6 fold increase in shoot number in Kn (5.0 µM) (Fig. 2) and problem of vitrification could also be overcome. In control, shoot multiplication was observed but shoot elongation occurred. Maximum shoot length i.e. 4.1 cm was evident in PGR free medium (control).

Table 5: Effect of cytokinins on shoot multiplication in *A. euchroma

PGRs (µM)		No. of shoots	Maximum shoot length (cm)
Kn	BAP		
1.0	-	6.0 ± 0.27	1.5 ± 0.12
2.5	-	19.0 ± 0.26	3.4 ± 0.19
5.0	-	**25.66 ± 0.38**	3.7 ± 0.15
10.0	-	12.66 ± 0.31	2.3 ± 0.16
-	2.5	15.0 ± 0.22	2.8 ± 0.05
-	5.0	22.66 ± 0.31	3.0 ± 0.34
-	10.0	**31.0 ± 0.11**	2.5 ± 0.16
-	-	5.0 ± 0	**4.1 ± 0.62**

*Data recorded after 30 days of culture
No. of replicates = 4
Initial no. of shoots = 4; initial shoot length = 1.0-1.3 cm
± = Standard deviation (mean)

Fig. 3 Shoots on TDZ 15.0 µM

In one experiment, varied response was observed when shoots with three leaves each were transferred to TDZ at 5.0, 10.0 and 15.0 µM concentrations. It was observed that number of shoots increased with increase in concentration of TDZ and within 15 days, more than six fold increase was observed at 15.0 µM TDZ (Table 6). However, at higher concentration (20.0 µM) the leaves became flattened and swollen. Although shoot number was more in 15.0 µM TDZ, but shoot length decreased (Fig. 3) with increase in TDZ concentrations being 1.24 in 15.0 µM TDZ concentration and 1.60 in 5.0 µM TDZ concentration. However, in control there was no increase in shoot number but shoot length was almost equivalent (1.55 cm) to that of 5.0 µM TDZ (1.6 cm).

Table 6: Effect of TDZ on shoot multiplication in *A. euchroma

Concentration (µM)	Mean number of shoots	Mean shoot length (cm)
5.0	12.44±0.57	**1.60±0.40**
10.0	15.66±1.52	1.33±0.28
15.0	**19.33±1.15**	1.24±0.33
Control	3.44±0.19	1.55±0.20

*Data recorded after 15 days of culture
No. of replicates = 3 (3 shoots per flask with 3 leaves)
Initial shoot length = 1.0-1.2 cm
± = Standard deviation (mean)

As is evident from above observations, Kn (5.0 µM) is most suited for shoot growth and elongation. So, for faster rates of multiplication shoots were initially multiplied on a TDZ (15.0 µM) containing medium for 15 days and were then sub-cultured on a medium containing Kn (5.0 µM) for shoot growth and elongation.

4.1.3 Regeneration

Different PGRs were used for assessing the regeneration potential of leaves obtained from *in vitro* shoot cultures, callus and shoots.

i) Leaf segments: It was noticed that when leaf segments were inoculated on BAP (10.0 µM) + IBA (5.0 µM) containing medium, greenish white, proliferating callus was obtained. When IBA was replaced by NAA (5.0 µM), callus induction was found to be delayed by 10 days and growth of callus was also less as compared to BAP and IBA containing medium (Table 7; Plate 1 a-c).

Table 7: Effect of PGRs on callus induction from *in vitro* leaf segments of *A. euchroma**

PGRs (µM)	Mean per cent response	Remarks
BAP (10) + IBA (5)	**75±50**	White, proliferating friable callus. Leaves remained green.
BAP (10) + NAA (5)	70±20	White friable callus very less proliferating. Leaves remained green.
Control	0±0	Leaves green without callusing
*Data recorded after 30 days of culture No. of replicates : 4 (3-5 leaf segments per petriplate) incubated in dark conditions in culture room ± = Standard deviation (mean)		

ii) Callus derived from leaves: The callus obtained was sub-cultured at 15 and 30 days interval on different concentrations and combinations of BAP and Kn along with 10.0 µM IBA (Table 8). The colour of the callus changed from greenish white to pink or brown with passage of time depending upon the concentration and combination of these PGRs. The callus formed was invariably friable except for BAP (5.0 µM, 10.0 µM) in

combination with IBA (10.0 μM) where compact and brownish pink callus was observed after 15 days. However, in none of the combinations, any sign of organogenetic response was observed.

Table 8: Effect of PGRs* and duration of sub-culturing on callus cultures of *A. euchroma*

PGRs (μM)		Callus response after days	
BAP	**KN**	**15**	**30**
-	-	Light green & creamish white	Brown coloured
0.1	-	Pink coloured	Brown coloured
0.05	-	Brownish pink	Brownish pink
1.0	-	Brownish pink	Brown coloured
0.5	-	Brownish pink, proliferating	Brownish pink
1.0	-	Brownish pink, proliferating	Brownish cream
3.0	-	Brownish pink with slight green colour	Brownish cream
5.0	-	Brownish pink, **compact**	Brown
10.0	-	Brownish pink, **compact**	Greenish white
-	0.1	Pinkish cream	Brown coloured, proliferating
-	0.05	Brownish pink, proliferating	Brownish pink, proliferating
-	1.0	Brownish dark pink, proliferating	Brown coloured
-	0.5	Brownish dark pink, proliferating	Brown coloured
-	1.0	Brownish cream, proliferating	Brown coloured
-	3.0	Brownish pink, proliferating	Brown coloured
-	5.0	Brownish pink, proliferating	Brownish pink, proliferating
-	10.0	Brownish pink, proliferating	Brown coloured
*IBA at 10 μM concentration was invariably used No. of replicates = 5 (incubated in dark conditions in culture room)			

iii) Complete leaves: Leaves turned brown and scanty brown coloured callus was formed, when picloram at 0.1, 0.5 and 1.0 μM concentrations were added to the medium. At 0.5 and 1.0 μM concentrations of picloram, root initiation was evident (Table 9) but after attaining a length of 15-20 mm, the tips of roots started turning brown, their growth stopped and ultimately these became dead. Even such roots did not grow further when excised along with a small lump of callus and cultured on same medium.

Table 9: Effect of picloram on *in vitro* leaves of *A. euchroma

Concentration (µM)	Mean per cent response (Callusing)	Remarks
0.1	83.33±23.57	Leaves turned brown, callus brown in colour, scanty
0.5	100±0	Scanty callus showing rhizogenesis
1.0	100±0	Scanty callus showing rhizogenesis
Control	-	No callusing occurred. Explants turned brown.

* Data recorded after 40 days of culture

No. of replicates = 3 (3 leaves per petriplate; incubated in dark conditions in culture room)

± = Standard Deviation (mean)

Another kind of PGR used was thidiazuron. At 1.0 µM and 2.5 µM concentrations, friable callus was obtained from the petiolar end of leaves but the leaves turned brown. At 5.0 µM concentration, the per cent response in terms of callusing was only 37.5 even after 40 days, but roots were seen emerging from the explant after 60 days of culture (Table 10; Plate 1 d and e). No organogenesis was observed from such calli on same medium or upon transfer to PGR free medium.

In all the experiments, in control, there was no callusing from leaf segments or complete leaves.

iv) Shoot cultures on TDZ (20.0 µM): It was observed that TDZ up to 15.0 µM was suitable for shoot multiplication, whereas on higher concentration i.e. 20.0 µM the leaves were vitrified and became flattened (Plate 2 a). However, on such leaves small protuberances emerged after 40 days (Plate 2 b) if the cultures remained on same medium. Based upon these observations an elaborate experiment was laid out.

Plate 1 a-e: Callus induction and proliferation of *A. euchroma*

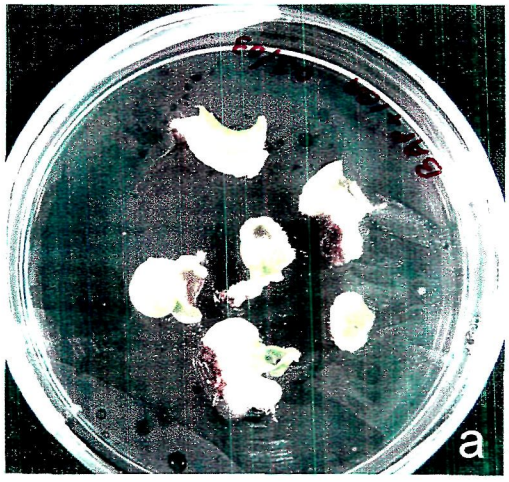
a) Callus induction on BAP (10.0 μM) + IBA (5.0 μM) after 5 days

b) Callus induction on BAP (10.0 μM) + NAA (5.0 μM) after 15 days

c) Callus proliferation on BAP (10.0 μM) + IBA (5.0 μM) after 40 days

d) Callusing on TDZ (1.0 μM) after 45 days

e) Callusing on TDZ (1.0 μM) after 45 days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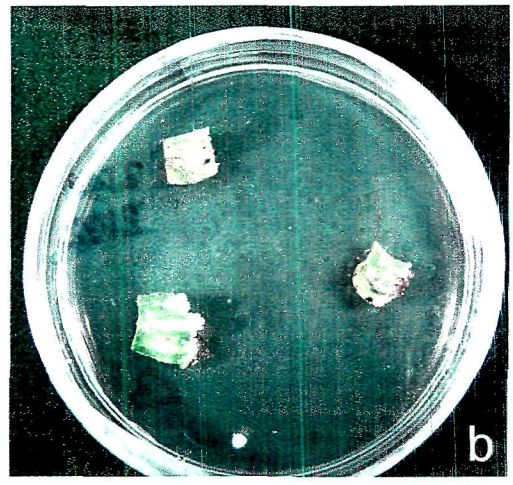
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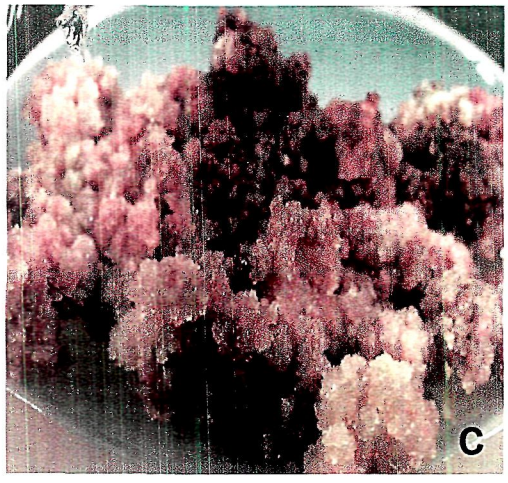
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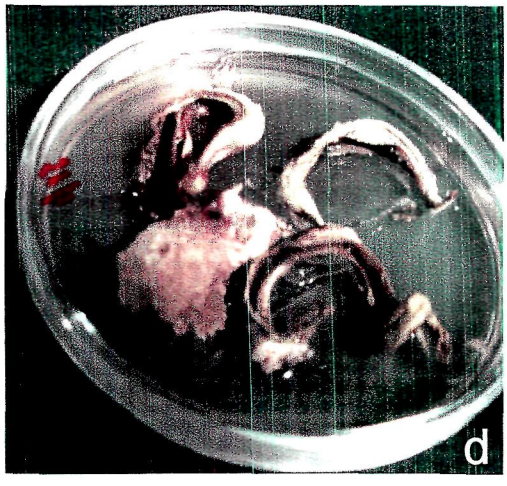
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Table 10: Effect of TDZ on *in vitro* leaves of *A. euchroma

Concentration (µM)	Mean per cent response (Callusing)	Days after	Remarks
1.0	54.15 ± 8.3	40	Friable callus induced from basal portion only and explants turned brown and enlarged. Callus is formed in lumps
2.5	43.75±12.5	40	Friable callus induced from basal portion only and explants turned brown and enlarged. Callus is formed in lumps
5.0	37.5±14.43	40	Friable callus induced from basal portion only and explants turned brown and enlarged. Callus is formed in lumps
		60	Root emergence from explant was also observed
Control	-	40	Leaves turned brown and no callusing

*No. of replicates : 4 (3-4 leaves per petriplate; incubated in dark conditions in culture room)

± = Standard Deviation (mean)

Shoots (1.0-1.5 cm long) with 3-4 leaves were allowed to grow on TDZ (20.0 µM) containing MS medium. It was observed that the protuberances formed on 20.0 µM TDZ did not grow further when left on the same medium or when transferred to PGR free medium and callus was produced from the base of shoots (Plate 2 c), when it was left for 60 days on the same medium (Table 11).

Table 11: Effect of TDZ (20.0 µM) on intact leaves of *A. euchroma

Days after	Type of response
20	Shoots became vitrified with swollen and flattened leaves
40	Protuberances from flattened leaf surface.
60	No growth of protuberances; callusing from base of shoots.
*No. of replicates = 4 (Plantlets with 2-3 leaves) Control: Plantlets on MS basal medium showed no such developments. ± = Standard deviation (mean)	

In one set of the experiments, the shoots showing such protuberances were transferred to lower concentrations i.e. 5.0 µM of TDZ and observations were recorded after 5, 10 and 20 days of culture (Table 12). There was not much difference after 5 days, however, after 10 days these protuberances grew further to form shoot buds (Plate 2 d), which subsequently formed shoots after 20 days (Plate 2 e and f). Per cent response in terms of shoot bud formation was low i.e. 44 and 7-15 shoot buds were formed from single explant. Conversion of shoot buds to shoots was 100 per cent. Here it is pertinent to mention, that all the leaves do not respond to form shoot buds. The response is also independent of leaf size or position. The identity of shoot buds was confirmed by histological evidence (Plate 3 a-d). Altered morphology with scattered vascular bundles was attributed to the effect of TDZ.

Table 12: After effect of TDZ (20.0 µM) on intact leaves of *A. euchroma when transferred to TDZ (5.0 µM)**

Days after transferring	Remarks / Response
5	Protuberances remained as such
10	Protuberances converted into shoot buds
20	Shoot development
*Shoots on TDZ (20.0 µM) were transferred to TDZ (5.0 µM) after 40 days	

Plate 2 a-f: Regeneration in *A. euchroma*

a) Swollen and vitrified leaves on TDZ (20.0 μM) after 20 days

b) Protuberances (arrow marked) on leaf surface on TDZ (20.0 μM) after 40 days

c) Callusing from the base of shoots on TDZ (20.0 μM) after 60 days

d) Formation of shoot bud (arrow marked) on TDZ (5.0 μM) after 10 days of transferring

e) Formation of shoot (arrow marked) on TDZ (5.0 μM) after 20 days of transferring

f) Formation of shoots (arrow marked) on TDZ (5.0 μM) after 20 days of transferring

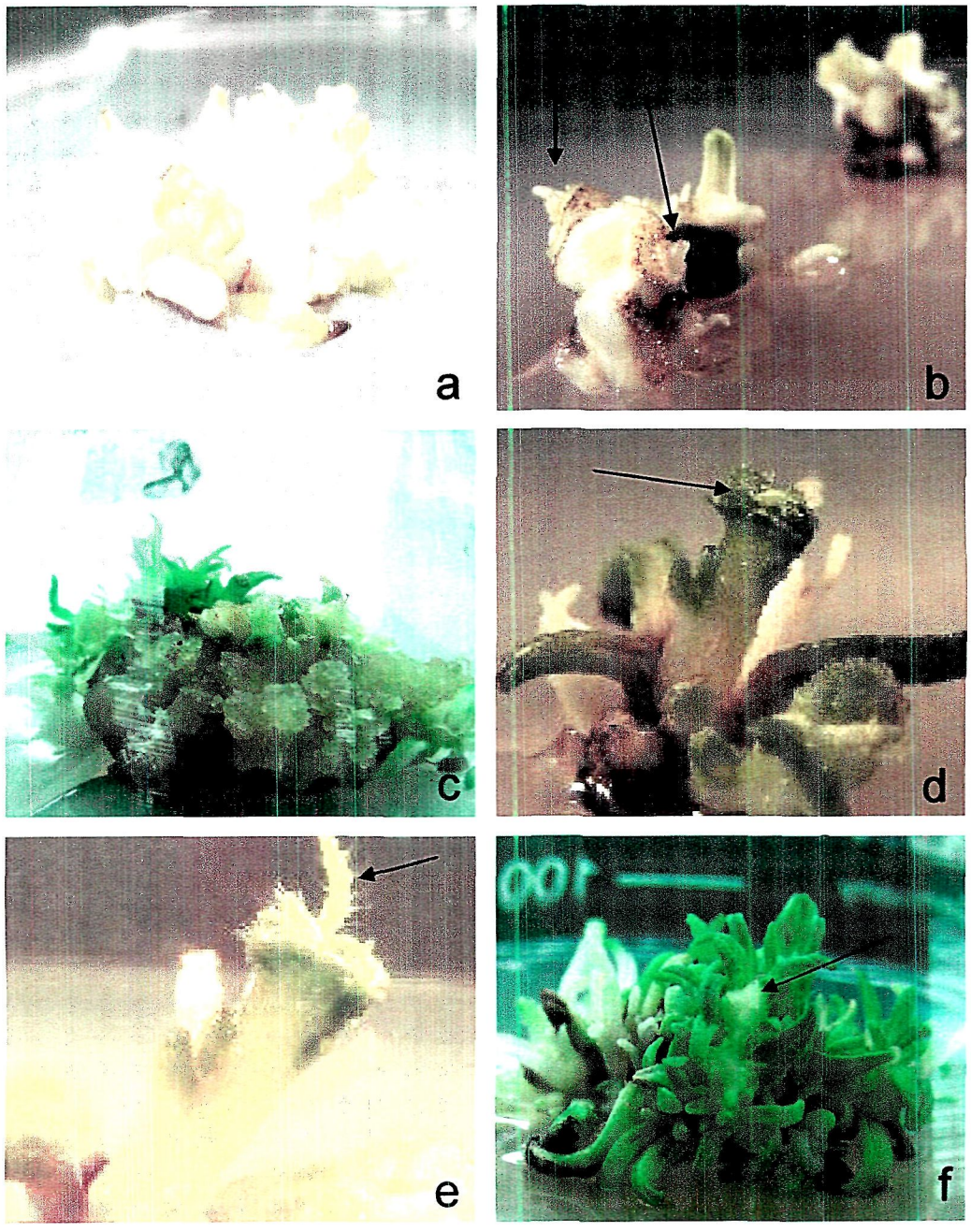
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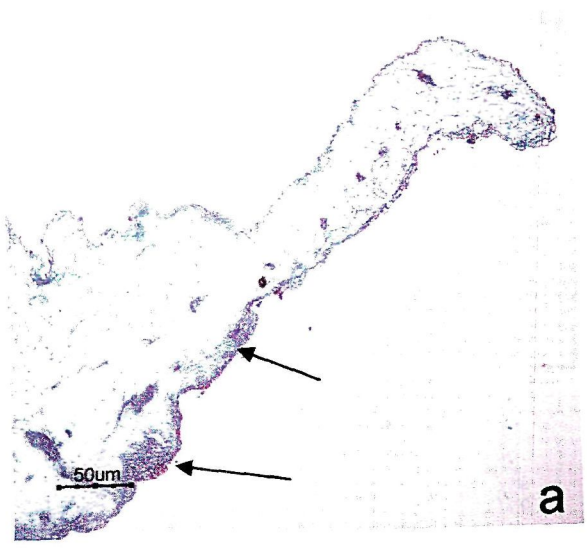
Plate 3 a-d: Histological studies in *A. euchroma*

a) A part of swollen leaf with protuberances (arrow marked)

b) Initiation of shoot bud. Note continuation of vascular strand with the mother explant

c) Protuberance from leaf margin

d) Shoot apex (sa) with subtending leaves (sl) and vascular strands (vs) (arrow marked)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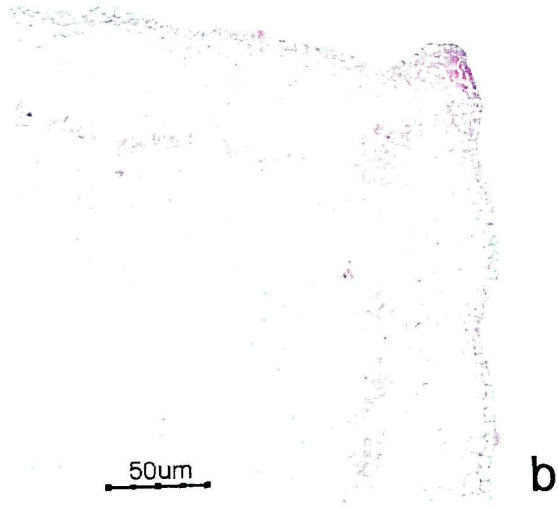
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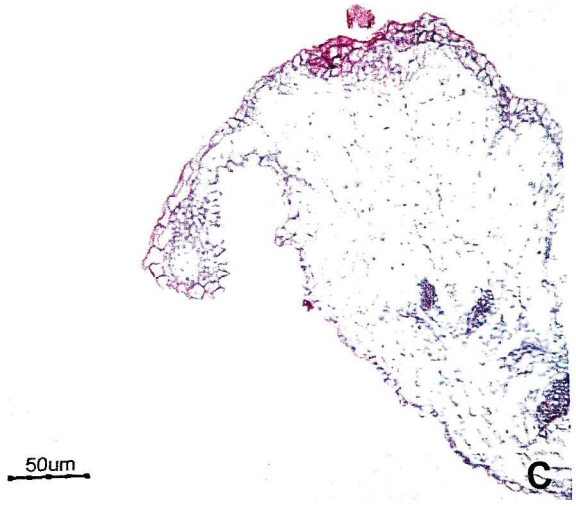
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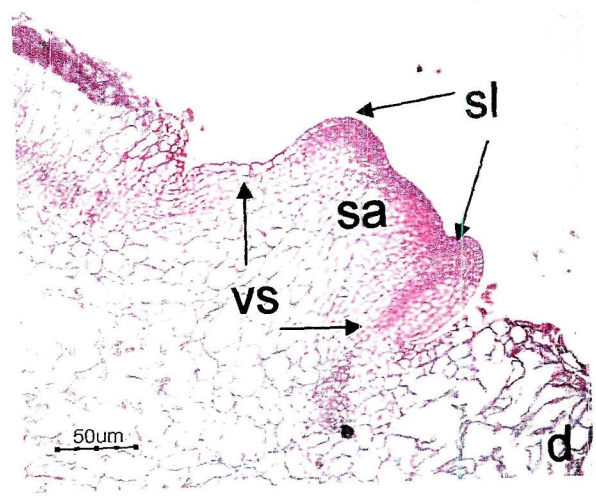
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v) **Callus derived from the base of shoots:** As mentioned above, callusing was induced from the base of shoots on TDZ (20.0 µM) after 60 days of culture. The callus thus produced when transferred to TDZ (5.0 µM) containing medium showed callus growth up to 30 days of culture, after which it declined. Of all 3 concentrations of $AgNO_3$, it was noticed that on 15.0 mg/l and 50.0 mg/l concentrations, the culture turned brown and no further growth occurred. However, on 35.0 mg/l $AgNO_3$ supplemented medium initially the callus turned brown after 30 days of culture but after 60 days, small, white, organized structures with shiny surface (Plate 4 a) started developing. These developed further to form globular structures but no further growth occurred even after 120 days of culture. Even their transfer to PGR free medium, did not help in further development of these structures. Histological studies revealed that these globular structures were comprised of densely cytoplasmic compact cells (Plate 4 b-d). These appeared to be embryo like structures. Further growth did not occur on any media combinations.

4.2 *S. rebaudiana*

4.2.1 Shoot multiplication:

i) **Raising of aseptic cultures and shoot proliferation:** Varied response of nodal segments was observed when inoculated on two different concentrations of BAP (Table 13). At 2.5 µM concentration, 2-3 shoots were formed from single axillary bud after 10 days of culture. The number of shoots increased to 5-6 and multiple shoots

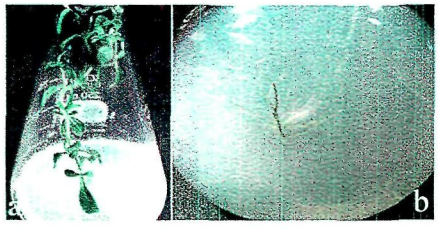


Fig. 4 a&b: Shoot proliferation and rooting on STS (2.5 µM)

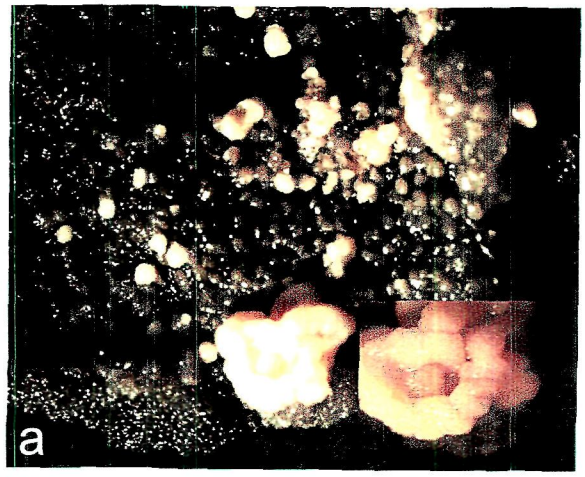
Plate 4 a-d: Somatic embryogenesis on $AgNO_3$ (35.0 mg/l)

a) Cream coloured somatic embryos on dark brown callus

b) Section of callus with somatic embryos

c) Heart shaped embryo

d) Section of callus showing pockets of densely cytoplasmic cells (Meristemoids)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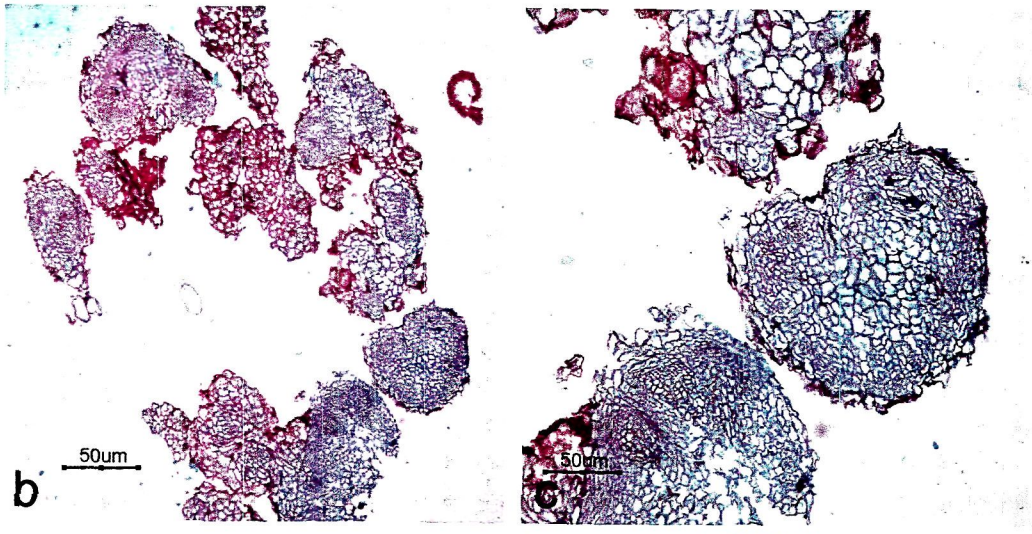
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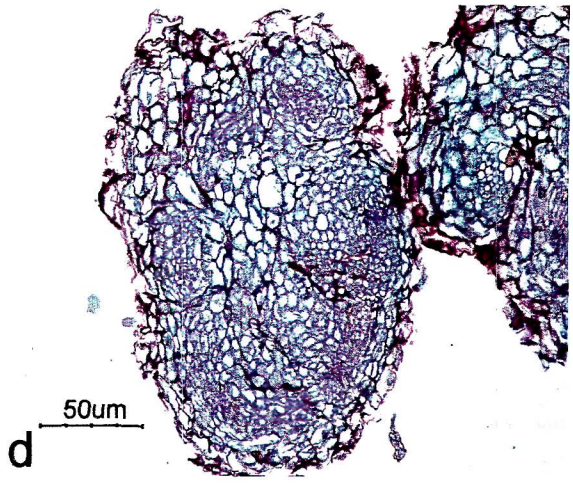
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were formed after 20 days. From cut end of shoots at the basal portion, callusing was also observed. However, on higher concentration of BAP i.e. 5.0 µM, axillary buds sprouted after 10 days but after 20 days, the leaves and no further growth occurred. On STS (2.5, 5.0 µM) supplemented medium, enlarged (1.0 cm wide), healthy and dark green leaves (Fig. 4 a) were obtained as compared to medium supplemented with BAP (2.5, 5.0 µM) (Table 13) where leaf width was only 0.5 cm.

Shoots proliferated further and dense rooting of shoots (Fig. 4 b) was also observed on the same medium within 30 days of initiation of cultures. Subsequently browning of leaves occurred on STS (5.0 µM) supplemented medium after 45 days. No callusing at the base of shoots was observed. However, on STS (2.5 µM), slight yellowing of leaves occurred if left on same medium for 60 days. This problem could be averted by sub-culturing on to fresh medium.

Table 13: Shoot multiplication in *S. rebaudiana

Concentration (µM)	Days after	Response
BAP (2.5)	10	2-3 shoots were formed
	20	5-6 shoots were formed. Callusing from cut ends
	45	Leaves started turning brown at tip portion
BAP (5.0)	10	2-3 shoots were formed
	20	Browning of leaves and stem portion
	45	-
STS (2.5)	10	-
	20	Green, enlarged, healthy leaves & shoots, with dense rooting, 15-18 in number with maximum length = 2.6 cm and minimum length = 0.5 cm
	45	-Do-
STS (5.0)	10	-
	20	Green, enlarged leaves, with roots 14-18 in number with maximum (2.6 cm) and minimum length (0.5 cm)
	45	Browning of leaves
Control	20	No increase in number of shoots

4.2.2 Regeneration studies

Different PGRs were used to assess regeneration potential of leaves from *ex vitro* as well as *in vitro* grown plants.

i) *Ex vitro* leaves: On BAP and 2, 4-D supplemented MS medium, only callusing was observed, although the extent varied depending upon the concentration and combination of these PGRs used (Table 14; Fig. 5). The leaves enlarged, curled and turned brown in all the PGR combinations. Creamish white and compact callus originated from cut portion of leaves. Callus was scanty and non-proliferative in 2, 4-D (2.5 µM), BAP (0.5, 1.0 µM), BAP (2.5 µM) + 2, 4-D (10.0 or 15.0 µM). In all the other PGR combinations, callus proliferated and covered the whole explant.

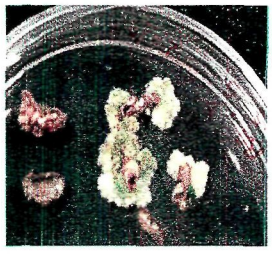

Fig. 5 Callusing on BAP (0.5 µM) and 2,4-D (5.0 µM)

The callus thus formed was transferred to lower concentrations of PGRs or to medium without PGRs, but no further response was observed in terms of organogenesis. It was observed that callus initiation was preponed by about 7 days in the presence of NAA alone or in combination with Kinetin (Table 15). Leaves enlarged and remained green at the time of callus inception. After about 3 months, white creamish callus turned green. The callus was friable as compared to compact callus in BAP and 2, 4-D combinations. In Kn (2.5 µM) alone or in combination with NAA (1.0, 2.5 and 5.0 µM), rhizogenesis in callus was noticed (Fig. 6). In most of the combinations, callus was proliferative but there was no sign of organogenesis even after prolonged culturing.

Fig. 6 Rhizogenic callus on Kn (2.5 µM) NAA (5.0 µM)

Table 14: Response of *ex vitro* leaf segments of *S. rebaudiana

PGRs (µM)		Mean % response (Callusing)	Remarks
BAP	**2, 4-D**		
0	2.5	50±0	Cream coloured, compact, scanty callus from cut portion of leaf
0	5	44.44±9.62	Cream, proliferating callus from lower side of leaf
0	10	72.21±9.62	-Do-
0	15	83.33±0	-Do-
0.5	0	22.22±19.24	Cream, scanty, compact, white callus
0.5	2.5	44.44±9.62	White coloured, proliferating, compact callus
0.5	5	77.77±9.62	White, compact callus surrounding the whole explant
0.5	10	90.43±8.25	Cream coloured, proliferating, compact callus from cut ends of leaves
0.5	15	83.33±0	-Do-
1	0	33.33±0	Scanty, cream, compact callus
1	2.5	44.44±9.62	Proliferating, cream, compact callus
1	5	72.22±19.24	-Do-
1	10	61.11±19.24	-Do-
1	15	83.33±0	-Do-
2.5	0	55.55±9.61	-Do-
2.5	2.5	100±0	-Do-
2.5	5	**100±0**	-Do-
2.5	10	77.77±25.45	Scanty, not proliferating, cream callus
2.5	15	83.33±28.86	-Do-
0	0	0±0	Leaves green in colour without callusing
*Data recorded after 15 days of culture No. of replicates = 3 (6-7 leaf segments per petriplate) ± = Standard deviation (mean)			

ii) ***In vitro*** **leaves:** Since size of leaves from *in vitro* shoots was smaller as compared to those taken from *ex vitro* source, complete leaves were taken rather than leaf segments. The source of these leaves was *in vitro* shoot cultures growing on BAP (2.5 μM) supplemented medium. As no organogenetic response was observed in lower concentration of 2, 4-D in the previous experiment from *ex vitro* leaves, a little higher concentration of 2, 4-D was employed i.e. 25.0 μM. BAP was used at 1.0 μM concentration and sucrose was also increased to 6 per cent and 12 per cent (Table 16). After 20 days of culture, callusing was observed in both the concentrations and combinations of these PGRs. After 60 days in 2, 4-D (25.0 μM) + BAP (1.0 μM) + sucrose (12%) smooth, well organized, shiny structures were formed (Plate 5 a and b) in the callus mass but the frequency of response was too low (6.25%).

On the other hand, only callusing took place on similar combination of these PGRs with sucrose (6%). In order to ascertain the identity of these structures, histological studies (Plate 5 c-f) were carried out. The transverse section of such callus masses showed groups of densely cytoplasmic cells, which were tightly packed and were surrounded by epidermal cells. Such meristemoids (Plate 5 e, f) may further develop to form somatic embryos. Presence of tracheids in callus (Plate 5 d) shows a step towards organogenesis.

Leaves from *in vitro* shoot cultures growing on STS (2.5 μM) were inoculated on silver nitrare (15.0, 35.0 mg/l). No response in terms of callusing or organogenesis was observed. The explants turned brown in colour.

Plate 5 a-f: Somatic embryogenesis from *in vitro* leaf segments of *S. rebaudiana*

a) & b) Formation of somatic embryos on BAP (1.0 µM) + 2, 4-D (25.0 µM) + sucrose (12%)

c) Histological section showing callus with organized structures

d) Tracheid formation in some callus masses

e) & f) Meristemoid formation in callus

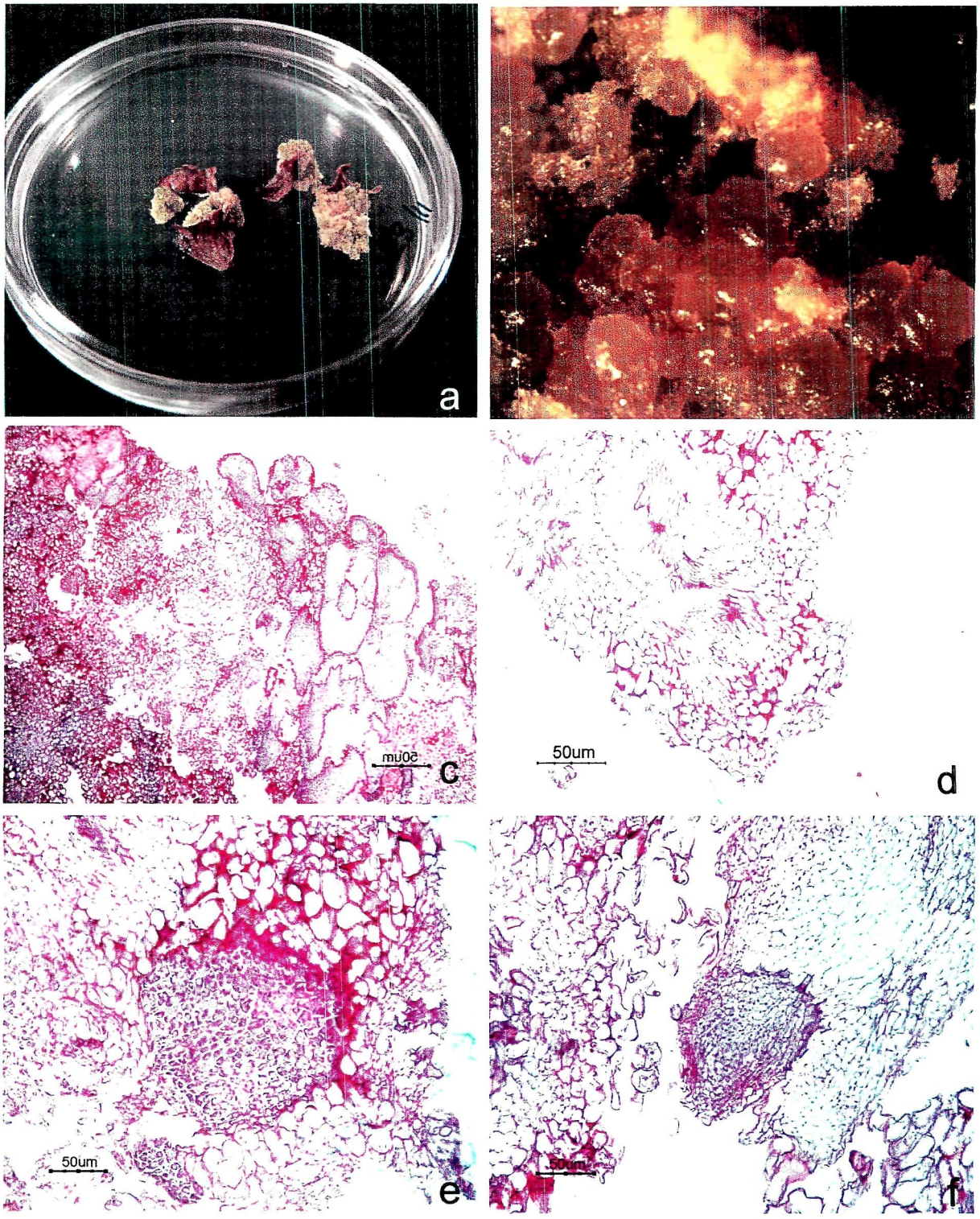
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Table 15: Effect of Kinetin and NAA on *ex vitro* leaf segments of *S. rebaudiana

PGRs (µM)		Mean % response (Callusing)	Days after	Remarks
Kn	NAA			
0	0	0±0	15	Leaves green in colour without callusing
0	1	100±0	7	White, friable, proliferating callus from cut portions of leaf and more from middle portion
0	2.5	0±0	15	Leaves green in colour without callusing
0	5	100±0	15	White, friable, proliferating callus from cut portions of leaf and more from middle portion
1	0	100±0	15	White, friable, scanty callus from cut portions of leaf
1	1	100±0	7	Friable, proliferating, white callus
1	2.5	100±0	7	-Do-
1	5	100±0	7	-Do-
2.5	0	100±0	15	Callus white, friable, scanty formed from midrib, upper and lower portion of leaf as well and rooting from middle portion of leaf
2.5	1	100±0	7	-Do-
2.5	2.5	100±0	7	-Do-
2.5	5	100±0	7	Roots white in colour from middle portion of leaf callus proliferating, white, friable and compact
5	0	100±0	7	No rooting and white, friable, scanty callus
5	1	100±0	15	-Do-
5	2.5	100±0	7	Callus white, friable, proliferating formed from midrib and veins also
5	5	100±0	7	-Do-

*No. of replicates = 3 (6 leaf segments per petriplate)
± = Standard deviation (mean)

Table 16: Effect of BAP and 2, 4-D on *in vitro* leaves of *S. rebaudiana

PGRs (µM)		Days after	Mean% response	Type of response	Remarks
BAP	2, 4-D				
1	10	20	100±0	Callus	Callus less formed and browning of leaves
		60	0±0	No somatic embryos	-
	25	20	100±0	Callus	More callus as compared to earlier combination and browning of leaves
		60	6.25±12.5	Somatic embryo like structures	Smooth, shiny structures
-	-	20	0±0	No callusing	Browning of leaves
*No. of replicates = 4 ± = Standard deviation (mean)					

iii) Shoot cultures on STS (2.5 µM): It was noticed that in one of the shoot cultures, on STS (2.5 µM) containing medium, protuberances emerged from margin of the leaf in touch with the medium (Plate 6 a and b). Such kind of response was noticed after prolonged culturing (beyond 60 days) on the same medium. The identity of these protuberances could not be ascertained, as these did not grow further. Besides, 2-3 shoot bud like structures started developing from leaf margins randomly in some of the cultures on same medium as described above. These leaves were not in touch with the medium. These were detached from the shoots and inoculated on BAP (2.5 µM) containing medium. It was observed that when leaves were transferred to BAP (2.5 µM) after 5-6 days of emergence of protuberances, these developed further to form shoot buds (Plate 6

c) followed by shoot formation (Plate 6 e and f). However, if such kinds of responding leaves are left as such and transferred later say 15 or 30 days, the growth ceased and no further development occurred (Table 17). However, the per cent response in terms of number of leaves responding and number of shoots of buds formed per leaf was too low (0.01%).

Table 17: Prolonged culturing of shoots on Silver Thiosulphate (STS; 2.5 μM) in *S. rebaudiana

Days after	Type of response
60	Formation of shoot buds on a leaf touching the medium. 2-3 shoot buds/leaf
75	Shoot bud formation on a leaf not touching the medium
100	-Do-
150	-Do-

iv) Nodal and internodal segments: Nodal segments placed horizontally on the medium containing silver nitrate (15.0, 35.0 mg/l) showed axillary bud sprouting after 7 days of culture. Internodal segments remained green but no regeneration or callusing response was evident.

v) Seeds and seedlings: Seed germination percentage was 57.14 on MS basal medium. The seeds germinated after about 8-10 days. The seedlings thus formed were used for regeneration studies on BAP (8.87 μM) and IAA (5.77 μM).

Different segments of the seedling i.e. shoot, hypocotyls and root portion remained green for about 30 days. Callus formation (Plate 7 a) occurred from the cut ends of shoot and hypocotyl segments of seedling but no response occurred from the root portion. After 30-35 days of culture small, shiny, green organized structures developed

Plate 6 a-e: Shoot bud and shoot development in *S. rebaudiana*

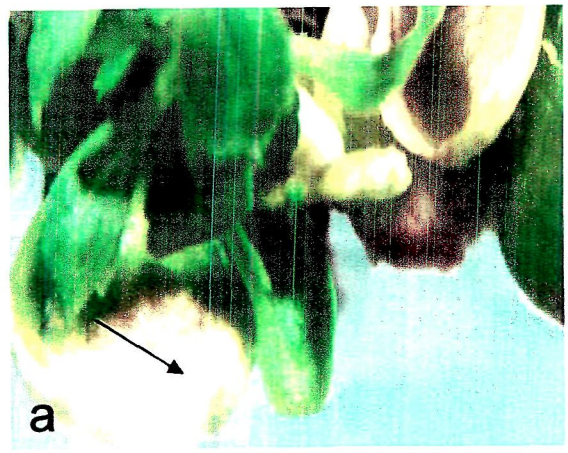
a) Protuberance (arrow marked) on intact leaf touching medium (STS; 2.5 μM)

b) Leaf excised with protuberance (arrow marked) on BAP (2.5 μM)

c) Further development of shoot buds on BAP (2.5 μM)

d) Shoots excised from leaf on BAP (2.5 μM)

e) Shoots proliferating on BAP (2.5 μM)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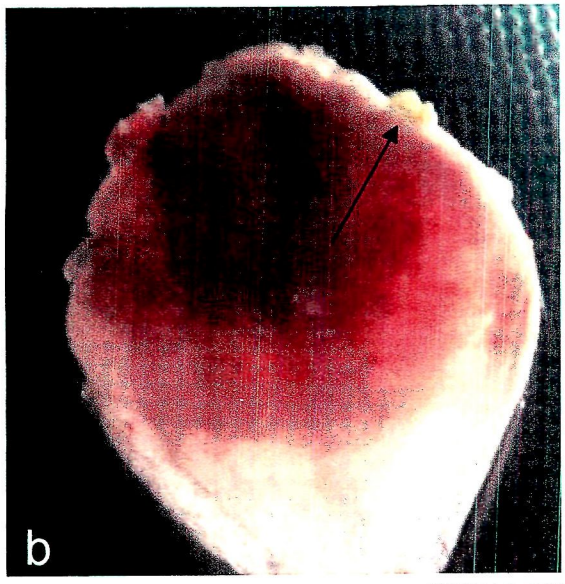
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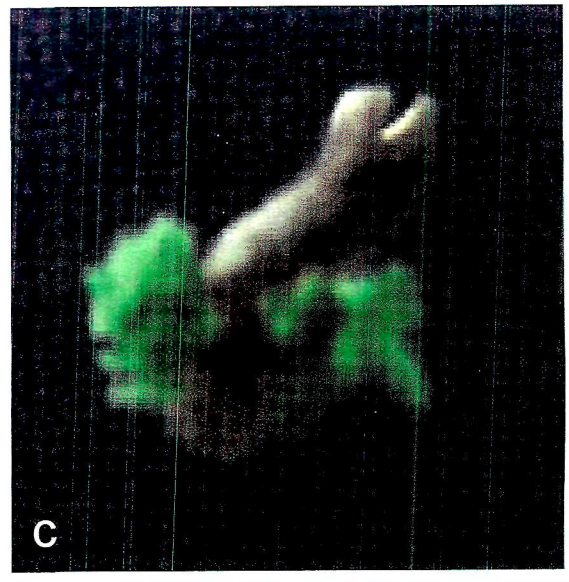
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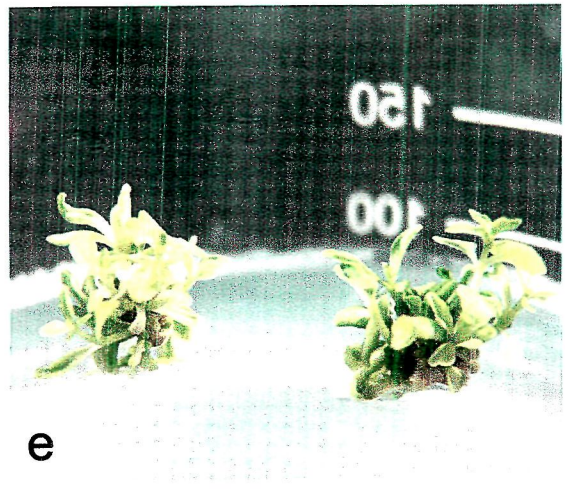
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(Plate 7 b) in the callus from cut end of hypocotyl. Histological studies revealed these to be somatic embryos (Plate 7 c-e). These did not grow further and development beyond heart shaped structure was arrested.

Plate 7 a-e: Somatic embryogenesis from seedlings

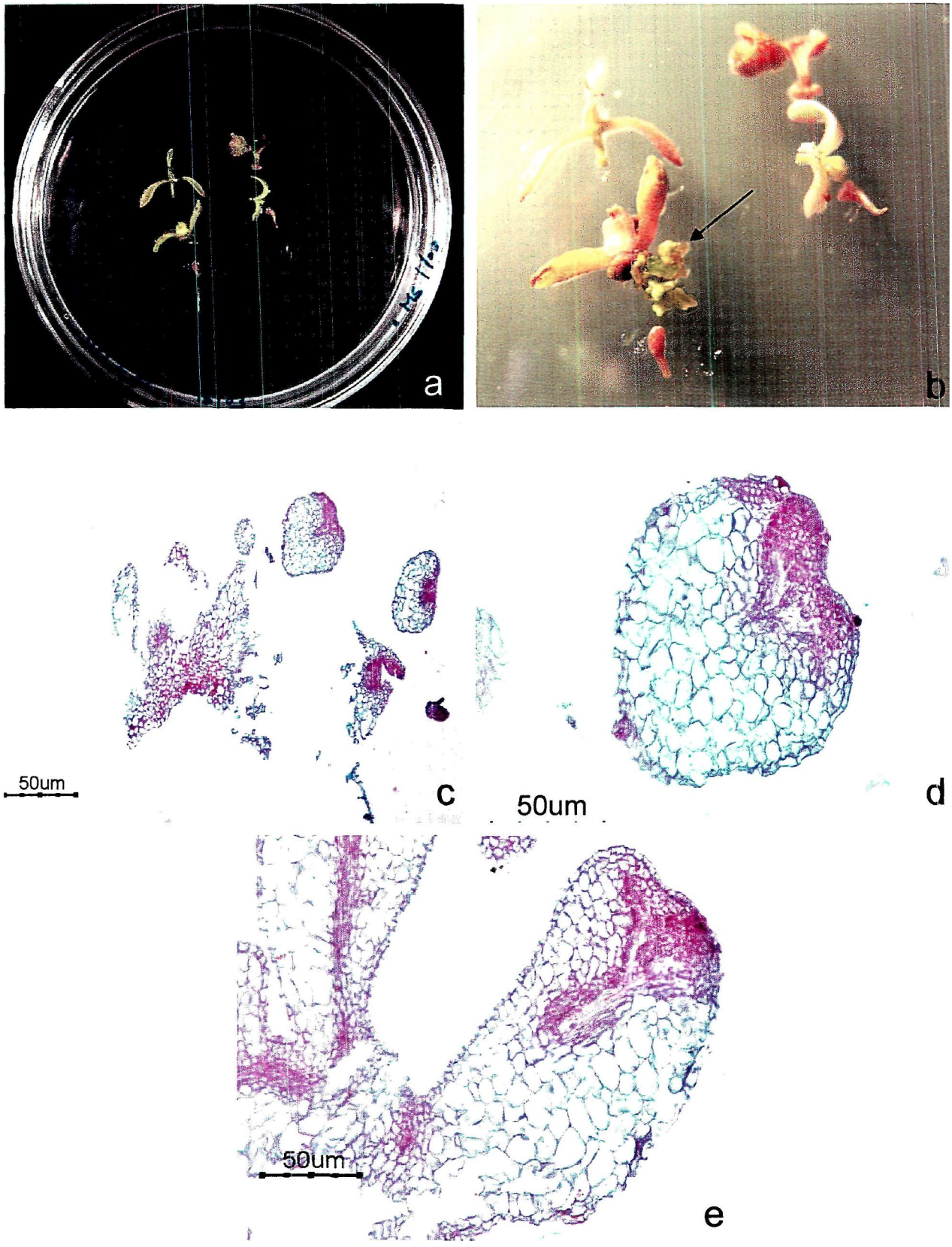
a) Callusing from cut end of hypocotyls (arrow marked) on BAP (8.87 μM) + IAA (5.77 μM)

b) Somatic embryos (arrow marked) from callus

c) Section showing callus mass with somatic embryos

d) Heart shaped somatic embryos with tracheid formation

e) Development of vascular strand from top of somatic embryo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Discussion

Chapter- V

DISCUSSION

Arnebia euchroma and *Stevia rebaudiana* are two medicinally important plants. Present studies were focused on raising aseptic cultures and assessing the regeneration potential of various explants. The results are discussed as follows:

5.1 *Arnebia euchroma*

5.1.1 Shoot multiplication

Contamination in shoot cultures occurring because of systemic bacteria was controlled by adding cefotaxime (100.0 mg/l) to the medium. Other antibiotics like streptomycin sulphate and kanamycin were also used but cefotaxime was best suited to control contamination without hampering the growth of cultures. Another problem was exudation of phenolics into the medium causing browning of cultures. In the present study, addition of PVPP (100.0 mg/l) was able to overcome this problem, however, Jiang *et al.* (2005) observed elimination of browning of shoots in *A. euchroma* with the addition of PVP (100.0 mg/l) to the medium. PVPP is considered to be better suited for overcoming the problem of phenolics exudation as it is insoluble and is not absorbed by the plant and hence does not interfere in plant metabolism.

Aseptic shoot cultures were raised on BAP (10.0 μM) + NAA (5.0 μM) containing medium. For shoot multiplication, cytokinins like BAP and Kn were used as cytokinins are known to promote shoot multiplication and proliferation (Kumar, 2002). After 30 days of culture, there was about 8 fold increase in shoot number on BAP (10.0 μM) and about 6 fold increase on Kn (5.0 μM) with maximum shoot length obtained on

the same concentration of Kn, but on BAP there was problem of vitrification. Vitrification is a morphological and physiological disorder frequently affecting both herbaceous and woody plants during *in vitro* vegetative regeneration (Lesham, 1983; Meira *et al.*, 1983). In apple, BAP availability in culture medium induced vitrification (Genkov and Ivanova, 1995).

For shoot multiplication, TDZ was also used. TDZ (15.0 μM) exhibited more than 6 fold increase in shoot number after 15 days, but shoot length decreased with increasing concentration of TDZ. At 20.0 μM concentration, there was problem of vitrification and shoot multiplication did not occur. TDZ, a substituted phenylurea is among the most active cytokinin-like substances and has been designated as a highly effective regulator of *in vitro* morphogenesis (Lu, 1993; Murthy *et al.*, 1998). It has been reported to produce greater number of shoots than other cytokinins and the number of reports indicating the use of TDZ for shoot regeneration in number of economically important plant species is increasing day by day (Huetteman and Preece, 1993; Li *et al.*, 2002; Vijaya Laxmi and Giri, 2003; Jiang *et al.*, 2005). To date the mode of action of TDZ is not clearly known but various studies speculate that it may modulate endogenous hormone activity, either directly or as a result of stress (Mok *et al.*, 1987; Hutchinson *et al.*, 1996; Murthy *et al.*, 1998). The decrease in shoot length with increasing concentration of TDZ in *A. euchroma* can be attributed with its high cytokinin activity due to increase in levels of cytokinins which inhibits the activity of cytokinin oxidase (Huetteman and Preece, 1993). Mok *et al.* (1982) reported the involvement of TDZ in cytokinin metabolism. TDZ being a potent inhibitor of cytokinin oxidase, causes the elevation of endogenous levels of cytokinins by preventing their breakdown to adenine

and adenosine (Chatfield and Armstrong, 1986; Hare and van Staden, 1994). TDZ has been used in axillary shoot proliferation of wide array of woody species such as in *Manihot esculenta* (Tunya *et al.* 1991), *Fraxinus Americana* (Yusnita *et al.* 1990).

Therefore, for shoot multiplication TDZ can be used but for shoot elongation the cultures may be transferred to medium lacking TDZ or on other PGRs as observed presently upon transfer of shoot cultures from TDZ to Kn containing medium.

5.1.2 Callus induction

Leaves produced greenish white, proliferating, friable callus on BAP (10.0 μM) + IBA (5.0 μM) and BAP (10.0 μM) + NAA (5.0 μM). Even after the sub-culturing of callus obtained on BAP + IBA, after 15 or 30 days on various concentrations of BAP and Kn with 10.0 μM IBA, no organogenesis was obtained. Only colour of the callus changed, from greenish white to pink or brown. Manjkhola *et al.* (2005) also obtained callus on BAP + IBA containing medium from leaf as well as root explants derived from seedlings, where colour and texture of callus varied according to the concentration of these PGRs. However, they could get simultaneous organogenesis and somatic embryogenesis from leaf derived callus with highest regeneration efficiency on IBA (2.5 μM) + BAP (2.5 μM), containing medium. Here it is important to mention that in their study, source of explant was different. They obtained leaf and root explants from aseptically raised seedlings whereas, in present study, leaves were used from *in vitro* shoot cultures. Complete leaves of *A. euchroma* produced callus on picloram or TDZ containing medium. Rhizogenesis was also observed on picloram (0.5, 1.0 μM) or TDZ (5.0 μM).

5.1.3 Regeneration

At higher concentration i.e. 20.0 µM of TDZ, flattened and swollen leaves were obtained after 20 days and callusing from the base of shoots took place after 60 days. Abnormal leaves with elongated hypocotyls and cotyledon were observed in *A. euchroma* by Jiang *et al.* (2005). Similar kind of effects induced by TDZ is reported in other plant species such as peanut (Murthy *et al.* 1995), chickpea (Murthy *et al.* 1996) and pigeon pea (Singh *et al.* 2003). Callus induction by high concentrations of TDZ in many woody species has been reported by Huetteman and Preece (1993). TDZ (1.0-50.0 µM) stimulated callus formation in black walnut (Huetteman, 1988) and similarly in silver maple, TDZ (more than equal to 1.0 µM concentration) induced callus growth (Ashby *et al.* 1987; Preece *et al.* 1991).

Vitrified intact leaves with protuberances formed on high concentration of TDZ (20.0 µM), when transferred to lower concentration i.e. 5.0 µM, showed shoot development after 20 days of transferring. Vitrification induced by TDZ has been observed in other plant species also, but formation of protuberances and then shoot development from such leaves has not been reported in other plant species. Also, shoot regeneration from intact leaves is the first report in *A. euchroma*. Regeneration of shoot buds was confirmed through histological studies.

Jiang *et al.* (2005) achieved shoot organogenesis in *A. euchroma* from hypocotyls and cotyledonary explants using TDZ. On TDZ (1.0 mg/l) more number of shoots regenerated from any of the explants used. About seven and six shoots were formed from cotyledon and hypocotyls explants on this concentration of TDZ. Since the seed raised progenies are heterogeneous, the cotyledon explants are not ideally suited to

obtain true to type plants. On the other hand, direct regeneration as evidenced through histological studies from intact leaves, as reported presently, ensures uniformity of plants thus produced. Altered morphology of leaves with scattered vascular bundles was observed when cultured on TDZ. Murthy *et al.* (1996) have also observed similar results from hypocotyledonary notch of chickpea cultured on TDZ containing medium. They observed abundant meristematic zones which elongated vertically and acquired defined shoot bud primordial shape.

The callus derived from the base of shoots after 60 days on TDZ (20.0 μM), when transferred to silver nitrate (35.0 mg/l) produced somatic embryo like structures. These were comprised of densely cytoplasmic compact cells. However, upon transfer to 5.0 μM TDZ, no such response was observed. This is the first report on the use of silver nitrate for somatic embryogenesis in *A. euchroma*. The process of somatic embryogenesis is inhibited by ethylene and silver ions are known to inhibit the action of ethylene. Therefore, it has been widely used either in the form of nitrate or thiosulphate (Biddington, 1992). Silver ion displaces an essential metal ion, perhaps copper from the active site of the metallo-protein receptor of ethylene (Beyer, 1976). Somatic embryogenesis has been reported in maize using silver ions in the form of thiosulphate (Veen, 1986) and in cereal callus (Purnhauser *et al.* 1987).

5.2 *Stevia rebaudiana*

5.2.1 Shoot multiplication

Shoots were proliferated from nodal segments on BAP (2.5 μM) with 5-6 shoots formed after 20 days but leaves were small sized (0.5 cm wide) and plant height was also short. Constantinovici and Cachita, (1997) too have used BAP for shoot

multiplication of *S. rebaudiana* using shoot apices as explants and found BAP more effective than Kinetin for shoot multiplication. But small leaves, thin and etiolated stem apical portions have been found to be associated with BAP (Morini *et al.* 2003).

Healthy, elongated shoots (3-4) with large (1.0 cm wide), dark green leaves were obtained along with rooting on the same medium on STS (2.5 μM) after 20 days.

Plants with stunted phenotype and smaller leaves are attributed with the release of ethylene in tightly sealed culture vessels. Ethylene is known to inhibit shoot culture growth (Hussey and Stacey, 1981; Perl *et al.* 1988) and leaf area production (Lentini *et al.* 1988) of *Solanum tuberosum* L. and *Brassica campestris* L., respectively. STS interferes with the physiological action of ethylene (Beyer, 1976) and thus increases leaf size in some of the plant species such as, in potato and improve leaf area production and nodal culture performance in papaya (Magdalita, 1997). Stimulation of lateral branch elongation has been obtained in *Hancornia speciosa* by the use of STS (Netto, 2001). STS also promoted shoot production in potato (Perl *et al.* 1988).

5.2.2 Callusing

Ex vitro leaf segments produced cream coloured, compact callus on varying concentration of BAP + 2, 4-D. Whereas, on some combinations of Kn + NAA supplemented medium, white coloured roots were formed and white, friable as well as compact callus formed on all combinations. Handro *et al.* (1977) also obtained vigorous callusing on BAP + 2, 4-D containing media from leaf discs and 1.0 cm long internodal segments.

5.2.3 Regeneration

From *in vitro* leaf segments, somatic embryos were obtained on BAP (1.0 μM) + 2, 4-D (25.0 μM) with increased sucrose concentration (12%). Histological studies indicated the presence of meristemoids and also presence of tracheids in callus tissue, a step towards organogenesis. Similar results were obtained by Bespalhok *et al.* (1993) in *S. rebaudiana* on BAP (1.0 μM) + 2, 4-D (10.0, 25.0 μM) but there was no intervening callus phase. Their results were not supported by histological studies. The induction of somatic embryogenesis at increased sucrose concentration could be attributed to the fact that sucrose acts as stressing agent thus causing organogenesis. Bespalhok and Hattori, (1997) too obtained somatic embryos in *S. rebaudiana* but from floret explants on 2, 4-D (9.05, 18.10 μM) and Kn (0-9.29μM) from embryogenic callus. They reported the presence of fibrillar network around proembryo which was autofluorescent. Sivaram and Mukundan (2003) have obtained shoot regeneration on BAP (8.87 μM) + IAA (5.71 μM) using from shoot apex, nodal and leaf explants.

An interesting observation was recorded in shoot cultures kept on STS (2.5 μM) for 60 days. A few leaves, may or may not in contact with the medium, showed development of adventitious shoot buds. When such leaves were detached from the shoots and grown on BAP (2.5 μM), the shoot buds developed further to form shoots. There is no such report of regeneration of shoot buds from intact leaves while still attached to the plant in *S. rebaudiana*. Plant regeneration has been reported using STS in maize (Veen, 1986) and in some potato cultivars (Hulme, 1992).

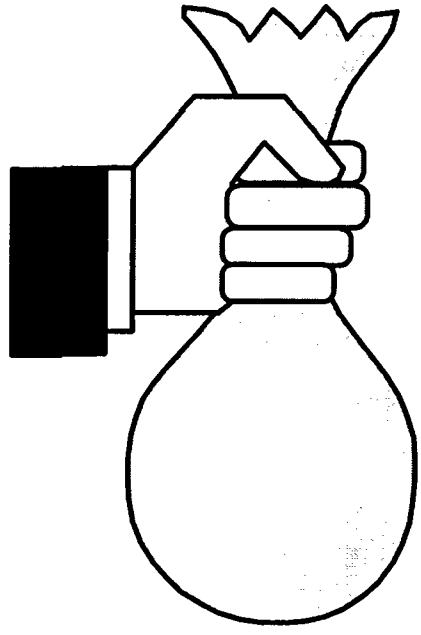
From the cut ends of hypocotyls of the seedling, somatic embryo like structures developed on BAP (8.87 μM) + IAA (5.77 μM) containing medium. Histological studies were carried out to ascertain the identity of such structures. Such

kind of response could be attributed to the age of explants as seedling explants are juvenile and respond better to various combinations of PGRs in the medium. Earlier, Yang and Chang (1979) observed regeneration of shoots from leaflets of 12-16 day old seedling on modified MS medium supplemented with BAP (2.0-10.0 mg/l). Rooting of shoots was obtained on PGR free medium. Park and Kim (2003) found regeneration from seed derived embryogenic callus on NAA (1.0 mg/l) and Kn (3.0 mg/l). However, embryogenic nature of callus was not verified through histological studies.

Some of the important findings of present work are:

1. TDZ can be used for shoot multiplication but for shoot growth and elongation Kn is suitable in *A. euchroma.*
2. For regeneration in *A. euchroma*, shoots with 5-6 leaves may be kept on higher concentration of TDZ (20.0 μM) and transferred to lower concentration i.e. 5.0 μM.
3. Silver nitrate be used for induction of somatic embryogenesis form callus in *A. euchroma.*
4. STS is promontory for shoot growth and bud regeneration in *S. rebaudiana.*
5. Seedling could be used for somatic embryo induction as the material is juvenile.

These experiments can be fine tuned further to standardize regeneration protocols. Since both of these are medicinally important plants, such protocols can be effectively used for transgenic development for enhanced production of desired components.



Summary

SUMMARY

The demand of medicinal plants is very high leading to their overexploitation from nature. Tissue culture methods provide an alternative to their conservation and multiplication. *A. euchroma* and *S. rebaudiana* have commercial importance due to their medicinal value. Shikonin and steviosides are the important compounds present in *A. euchroma* and *S. rebaudiana*, respectively.

The present studies were carried out with the objective of raising aseptic cultures and evaluating the regeneration potential of various explants. Explants both from *in vitro* shoot cultures and *ex vitro* grown plants were used in the present study.

Various PGRs and additives like IAA, IBA, NAA, Picloram, BAP, TDZ, Kinetin, 2, 4-D, silver nitrate, silver thiosulphate were added to Murashige and Skoog (1962) medium. Selection of these PGRs was based upon literature reports and previous work being carried out in our laboratory.

6.1 *A. euchroma*

Aseptic shoot cultures were raised on BAP (10.0 μM) + NAA (5.0 μM) containing medium. Problem of systemic bacteria and browning of medium due to phenolic exudation was overcome by the addition of cefotaxime (100.0 mg/l) and PVPP (100.0 mg/l) to the medium. Although, about 8 fold increase in shoot number after 30 days was achieved in BAP containing medium, but there was a problem of vitrification. In TDZ (15.0 μM) shoots multiplied (more than 6 fold in 15 days) but shoot elongation did not occur. However, in Kn (5.0 μM) both shoot growth and elongation was evident. Therefore, multiple shoots obtained in TDZ were transferred to Kn (5.0 μM) for further elongation.

In excised leaves, regeneration was not observed on any of the media combinations. Shoot cultures growing on TDZ (20.0 μM) formed protuberances from intact, vitrified, swollen leaves after 40 days and later on produced shoots when transferred to lower concentration of TDZ (5.0 μM) after 20 days of transfer. Histological studies revealed these to be shoot buds.

When the shoots were left on TDZ (20.0 μM), callusing from the base of shoots occurred after 60 days. This callus when transferred to silver nitrate (35.0 mg/l), formed cream coloured, smooth, somatic embryo like structures as was evident through histological studies.

6.2 *S. rebaudiana*

BAP (2.5 μM) and STS (2.5 μM) were used to raise aseptic shoot cultures and to achieve shoot multiplication. On BAP (2.5 μM), 5-6 short shoots with small sized leaves were formed after 20 days, whereas on STS (2.5 μM), 3-4 healthy, elongated shoots with large leaves and rooting were obtained after 20 days.

Callus derived from *in vitro* leaves showed somatic embryogenesis on BAP (1.0μM) + 2, 4-D (25.0 μM) with increased sucrose concentration (12%). The identity of these structures was revealed by histological studies.

When the shoots were cultured for long duration i.e. 60 days on STS (2.5 μM), stunted shoot growth with formation of shoot buds from the margin of intact leaf was obtained. These leaves when excised from the plants, produced shoots on BAP (2.5 μM) containing medium. Such shoots, when separated from the leaf, proliferated on BAP (2.5 μM).

The cut end of hypocotyls of segmented seedlings showed somatic embryogenesis on BAP (8.87 μM) + IAA (5.77 μM) containing medium. Histological studies confirmed their identity.

Taking into consideration, the important leads with respect to regeneration potential of different explants in both the species, further experiments are required to standardize the regeneration protocols. Such protocols can be used for mass multiplication and transgenic production in order to get desired components.

Literature Cited

LITERATURE CITED

Acuna, I., Nepovim, A. and Valicek, P. 1997. Micropropagation of plants *Stevia rebaudiana* Bertoni *in vitro* and content of Stevioside in leaves after application of growth regulators under field conditions. Agricultura Tropica et Subtrpoica 30:51-60.

Akita, M., Shigeoka, T., Koizumi, Y. and Kawamura, M. 1994. Mass propagation of shoots of *Stevia rebaudiana* using a large scale bioreactor. Plant cell rep. 13(3/4): 180-183.

Ashby, W.C., Preece, J.E., Huetteman, C.A., Bresnan, D.F. and Roth, P.L. 1987. Silver maple tree improvement for biomass production. pp. 6-23 *In* N.D. Fargo (ed) Proc 5th North Central Tree improvement Conf. 1987.

Bespalhok, F.J.C. and Hattori, K. 1997. Embryogenic callus formation and histological studies from *Stevia rebaudiana* (Bert.) Bertoni floret explants. Revista Brasileira de Fisiologia Vegetal 9(3): 185-188.

Bespalhok, F.J.C., Hashimoto, J.M. and Vieira, L.G.E. 1993. Induction of somatic embryogenesis from leaf explants of *Stevia rebaudiana*. Revista Brasileira de Fisiologia Vegetal 5(1): 51-53.

Bespalhok, F.J.C., Vieira, L.G.E. and Hashimoto, J.M. 1992. Factors influencing the in vitro micropropagation of axillary shoots of Stevia rebaudiana (Bert.) Bertonil. Revista Brasileira de Fisiologia Vegetal 4(1): 59-61.

Beyer, E. 1976. Apotent inhibitor of ethylene action in plants. Physiologia Plantarum 58: 268-271.

Biddington, N.L. 1992. The influence of ethylene in plant tissue culture. Plant Growth Regulation 11: 173-187.

Chatfield, J.M. and Armstrong, D.J. 1996. Regulation of cytokinin oxidase activity in callus tissues of *Phaseolus vulgaris* L. cv. Great Northern. Plant Physiology 80: 493-499.

Chen, S.Y., Hou, S.S., Zhang, J., Li, X.M., Ye, H.C. and Li, G.F. 1994. Progressive scale-up culture of *Arnebia euchroma* (Royle) Johnst cells. Journal of Plant Resources and Environment 3(3): 27-31.

Chen, X., Oppenheim, J. and Zack Howard, O.M. 2001. Shikonin: a component of anti-inflamatory Chinese herbal medicine, selectively blocks chemokine binding to CC chemokinereceptor-1. Int. J. Immunopharmacol 1: 229-236.

Constantinovici, D. and Cachita, C.D. 1997. Aspects of in vitro multiplication in *Stevia rebaudiana* Bert. Cercetari Agronomice in Moldova 30(4): 80-86.

Das, K., Dang, R., Khanam, S., Shivananda, B.G. and Rajasekharan, P.E. 2005. In vitro methods for production of stevioside from *Stevia rebaudiana*. Indian Journal of Natural Products 21(1): 14-15.

Dong, J.W., Ye, H.C., Wu, X., Li, G.F., Wu, Z.R., Gu, L.M. and Chen, J.L. 1993. Studies on cell suspension culture and fermentation culture of *Arnebia euchroma*. Acta Botanica Sinica 35(1): 57-61.

Felippe, G.M. and Lucas, N.M.C. 1971. Estudo da viabilidade dos frutos de *Stevia rebaudiana* Bert. Hoehnea 1:95-105.

Felippe, G.M., Lucas, N.M.C., Behar, L. and Oliveira, M.A.C. 1971. Observacoes a respeito de germinacao de *Stevia rebaudiana* Bert. Hoehnea 1:81-93.

Ferreira, C.M. and Handro, W. 1988a. Production, maintenance and plant regeneration from cell suspension cultures of *Stevia rebaudiana* (Bert.) Bertoni. Plant Cell Reports 7(2): 123-126.

Ferreira, C.M. and Handro, W. 1988b. Micropropagation of *Stevia rebaudiana* through leaf explants from adult plants. Planta Medica 54(2): 157-160.

Fukui, H., Hasan, A.F.M.F. and Kyo, M. 1999. Formation and secretion of a unique quinone by hairy root cultures of *Lithospermum erythrorhizon*. Photochemistry 51: 511-515.

Ge, F., Wang, X.D. and Wang, Y.C. 2003. Advances in studies on medicinal *Radix Arnebia Seu Lithospermi*. Chin. Trad. Herbal Drugs 34: 7-10.

Genkov. T. and Ivanova, I. 1995. Effect of cytokinins-active phenylurea derivatives on shoot multiplication, peroxidase and superoxide dismutase activities of *in vitro* cultured carnation. Bulg. J. Plant Physiol. 21(1): 73-83.

Handro, W., Hell, K.G. and Kerbauy, G.B. 1977. Tissue culture of *Stevia rebaudiana*, a sweetening plant. Planta Medica 32(2): 115-117.

Harborne, J.B. and Baxter, H. 1996. Dictionary of plant toxins. New York: John Wiley and Sons

Hare, P.D. and van Staden, J. 1994. Cytokinin oxidase: biochemical feature and physiological significance. Physiologia Plantarum 91: 128-136.

Huetteman, C.A. 1988. *In vitro* culture of *Juglans nigra* L.: Micropropagation from embryonic axes and forcing of adult quiescent stems. MS Thesis. Southern Illinois University, Carbondale.

Huetteman, C.A. and Preece, J.E. 1993. Thidiazuron: a potent cytokinin for woody plant tissue culture, Plant Cell, Tissue and Organ Culture 33: 105-119.

Hulme, J.S., Higgins, E.S. and Shields, R. 1992. An efficient genotype-independent method for regeneration of potato plants from leaf tissue. Plant Cell Tissue and Organ Culture 31(2) : 161-167.

Hussey, G. and Stacey, N.J. 1981. *In vitro* propagation of potato (*Solanum tuberosum* L.). Ann. Bot. 48: 787-796.

Hutchinson, M.J., Murch, S.J. and Saxena, P.K. 1996. Morphoregulatory role of thidiazuron: evidence of the involvement of endogenous auxin in thidiazuron-induced somtic embryogenesis of geranium (*Pelargonium* X *hortorum* Bailey). Journal of Plant Physiology 149: 573-579.

Ji, Q.L. and Wang, W.G. 2001. Asexual propagation of *Arnebia euchroma* and exploration of hereditary stability in regenerated plantlets. Plant Physiology Communications 37(6): 499-502.

Leshem, B. 1983. Growth of carnation meristems *in vitro*: anatomical structure of abnormal plantlets and the effect of agar concentration in the medium on their formation. Annals of Botany 413-415.

Li, W., Gao, H.H., Lu, R., Guo, G.Q. and Zheng, G.C. 2002. Direct plantlet regeneration from the tuber of *Stachys sieboldii*. Plant Cell, Tissue and Organ Culture 71: 259-262.

Lu, C.Y. 1993. The use of thidiazuron in tissue culture. *In Vitro* Cellular and Developmental Biology-Plant 29: 92-96.

Magdalita, P.M., Godwin, I.D., Drew, R.A. and Adkins, S.W. 1997. Effect of ethylene and culture environment on development of papaya nodal cultures. Plant Cell, Tissue and Organ Culture 49: 93-100.

Maharik, N.T. and El Gengaihi, S.E. 2003. Micropropagation of *Stevia rebaudiana* Bertoni. Egyptian Journal of Horticulture 30(1/2): 125-134.

Manjkhola, S., Dhar, U. and Joshi, M. 2005. Organogenesis, Embryogenesis, And Synthetic Seed Production in *Arnebia euchroma* - A Critically Endangered Medicinal Plant of the Himalaya. In vitro cell. Dev. Biol.-Plant 41: 244-248.

Meira, Z., Meir, G. and Halevy, A.H. 1983. Factors influencing the production of hardened glaucous carnation plantlets *in vitro*. Plant Cell Tissue and Organ Culture 2: 55-65.

Miyagawa, H., Fujioka, N., Kohda, H., Yamasaki, K., Taniguchi, K. and Tanaka, R. 1986. Studies on the tissue culture of *Stevia rebaudiana* and its components. (II) Induction of shoot primordia. Planta Medica 4: 321-323.

Mok, M.C., Mok, D.W.S., Armstrong, D.J., Shudo, K., Isogai, Y and Okamoto, T. 1982. Cytokinin activity of N-phenyl-N'-1,2,3-thidiazol-5-yl urea (thidiazuron). Phytochemistry 21: 1509-1511.

Mok, M.C., Mok, D.W.S., Turner, J.E. and Mujer, C.V. 1987. Biological and biochemical effects of cytokinin-active phenylurea derivatives in tissue culture systems. HortScience 22(6): 1194-1197

Morini, S., Fiaschi, G., Andolfi, L. and Macchia, M. 2003. In vitro propagation of *Stevia rebaudiana* Bertoni: results with different genotypes. Agricoltura Mediterranea 133(2):117-123.

Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiologia Plantarum 15: 473–497.

Murthy, B.N.S., Murch, S.J. and Saxena, P.K. 1995. Thidiazuron induced somatic embryogenesis in intact seedlings of peanut (*Arachis hypoaea* L.): endogenous growth regulator levels and significance of cotyledons. Physiol Plant 94: 268-276.

Murthy, B.N.S., Murch, S.J. and Saxena, P.K. 1998. Thidiazuron: a potent regulator of *in vitro* plant morphogenesis. *In Vitro* Cellular and Developmental Biology-Plant 34: 267-275.

Murthy, B.N.S., Victor, J., Singh, R.P., Fletcher, R.A. and Saxena, P.K. 1996. *In vitro* regeneration of chickpea (*Cicer arietinum* L.): Stimulation of direct organogenesis and somatic embryogenesis by thidiazuron. Plant Growth Regulation 19: 233-240.

Nakamura, S. and Tamura, Y. 1985. Variation in the main glycosides of Stevia (Stevia rebaudiana Bertoni). Jpn J. Trpo. Agric. 29: 109-116.

Nepovim, A. and Vanek, T. 1998. In vitro propagation of *Stevia rebaudiana* plants using multiple shoot culture. Planta Medica 64(8): 775-776.

Netto, A.B.P. 2001. Effect of inhibitors of ethylene biosynthesis and signal transduction pathway on the multiplication of *in vitro*-grown *Hancornia speciosa*. Plant Cell, Tissue and Organ Culture 66: 1-7.

Park, K.H. and Kim, K.M. 2003. Factors Faaecting Plant Regeneration in the Culture of Different explants of *Stevia (Stebia rebaudiana* Bertoni). Korean Journal of Plant Biotechnology 30(2): 151-154.

Perl, A., Aviv, D. and Galun, E. 1988. Ethylene and *in vitro* culture of potato: Suppression of ethylene generation vastly improves protoplast yield, plating efficiency and transient expression of an alien gene. Plant Cell Rep 6: 1-4.

Preece, J.E., Huetteman, C.A., Ashby, W.C. and Roth, P.L. 1991. Micro-and cutting propagation of silver maple. I. Results with adult and juvenile propagules. J. Amer. Soc. Hort. Sci. 116: 142-148.

Purnhauser, L., Medgyesy, P., Czako, M., Dix, P.J. and Maston, L. 1987. Stimulation of shoot regeneration in *Triticum aestivum* and *Nicotiana plumbaginifolia* Viv. Tissue cultures using the ethylene inhibitor, silver nitrate. Plant Cell Rep 6: 1-4.

Purohit, S.S. and Vyas, S.P. 2004. Medicinal Plant Cultivation – A Scientific Approach. Agrobios (India) 1-9 pp.

Sakaguchi, M. and Kan, T. 1982. Japanese researches on *Stevia rebaudiana* (Bert.) Bertoni and stevioside. Ci Cult. 34: 235-248.

Sharma, R. 2003. Medicinal plants of India- an encyclopaedia. Daya Publishing House. 25 p.

Singh, N.D., Sahoo, L., Sarin, N.B. and Jaiwal, P.K. 2003. The effect of TDZ on organogenesis in pigeonpea (*Cajanus cajan* L. Millsop). Plant Sci. 164: 341-347.

Sivaram, L. and Mukundan, U. 2002. Feasibility of commercial micropropagation of *Stevia rebaudiana* in India. Journal of Tropical Medicinal Plants 3(1): 97-103.

Sivaram, L. and Mukundan, U. 2003. In vitro culture studies on *Stevia rebaudiana*. In vitro Cellular and Developmental Biology-Plant 39(5): 520-523.

Smitha, P.S., Nazeem, P.A., Thomas, J., Keshavachandran, R. and Girija, D. 2005. Micropropagation for mass multiplication of the important medicinal sweet herb- *Stevia rebaudiana*. Journal of Medicinal and Aromatic Plant Sciences 27(2): 247-252.

Tabata, M. and Fujita, Y. 1985. Production of shikonin by plant cell cultures. In: Zaitlin, M., ed. Biotechnology in plant Science. New York: Academic Press, 207p.

Tabata, M., Misukami, H. and Hiraka, N. 1974. Pigment formation in callus culture of *Lithospermum erythrorhizon*. Phytochemistry 13: 927-932.

Tamura, Y., Nakamura, S., Fukui, H. and Tabata, M. 1984. Clonal propagation of *Stevia rebaudiana* Bertoni by stem-tip culture. Plant cell rep. 3: 183-185.

Tripathi, L. and Tripathi, J.N. 2003. Role of biotechnology in medicinal plants. Trop J Pharm Res. 2(2): 243-253.

Tunya, G.O., Skirvin, R.M., Gerber, J. and Splitstoesser, W. 1991. Systems for the proliferation of cassava (*Manihot esculenta* Crantz.) *in vitro*. HortScience 26: 756.

Veen, H. 1986. Silver thiosulfate: an experimental tool in plant science. Acta Hortic. 20: 211-224.

Vijaya Laxmi, G. and Giri, C.C. 2003. Plant regeneration via organogenesis from shoot base- derived callus of *Arachis stenosperma* and *A. villosa*. Current Science 85(11): 1624-1628.

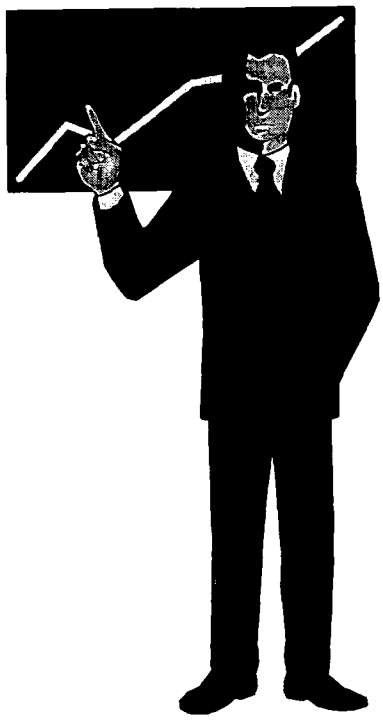
Xiao, J., Yang, Y.G., Guo, Y.M., Huang, Q.N. and Guo, Z.C. 2004. Advances in tissue culture of *Arnebia euchroma*. Acta Botanica Boreali Occidentalia Sinica 24(8): 1560-1564.

Yang, N.B. 1981. In vitro clonal propagation of twelve plant species. Acta Botanica Sinica 23(4): 284-287.

Yang, Y.W. and Chang. W.C. 1979. In vitro plant regeneration from leaf explants os Stevia rebaudiana Bertoni. Zeitschrift fur Pflanzenphysiologic 93(4): 337-343.

Yusinata, S., Geneve, R.L. and Kester, S.T. 1990. Micropropagation of white flowering eastern redbud (*Cercis Canadensis* var. alba L.). J. Environ. Hort. 8: 177-179.

Zbughin, G., Sturzu, T.M. and Constantinovici, D. 2002. Some aspects about "*in vitro*" multiplication process of *Stevia rebaudiana* (Bertoni) Hemsl. (Asteraceae). Analele Stiintifice ale Universitatii 'Al I Cuza' din Iasi Serie Nova Sectiunea-II Biologic Vegetala 48:43-52.



Appendices

APPENDIX-I

Composition of Murashige and Skoog (1962)*

Components	Concentration (mg/l)
NH_4NO_3	1650
KNO_3	1900
$CaCl_2.2H_2O$	440
$MgSO_4.7H_20$	370
KH_2PO_4	170
H_3BO_3	6.2
$MnSO_4.4H_20$	22.3
$ZnSO4.7H_20$	8.6
KI	0.83
$Na_2MoO_4.2H_2O$	0.25
$CuS0_4.5H_2O$	0.025
$CoCl_2.6H_20$	0.025
$FeSO_4.7H_20$	27.8
$Na_2EDTA.2H_20$	37.3
Meso-inositol	100
Pyridoxine-HCl	0.5
Thiamine-HCl	0.1
Nicotinic acid	0.5
Glycine	2.0

*Murashige T & Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15: 473-479

APPENDIX-II

*Stock solutions for MS basal medium

Components	Concentration (mg/500ml)	Volume of stock per litre of medium (ml)
MS stock		
$CaCl_2.2H_2O$	22000	10
$MgSO_4.7H_20$	18500	
KH_2PO_4	8500	
$Na_2EDTA.2H_20$	1862.5	
$FeSO_4.7H_20$	1392.5	
Nitrate stock		
NH_4NO_3	82500	10
KNO_3	38000	25
ME stock		
H_3BO_3	310	10
$MnSO_4.4H_20$	1115	
$ZnSO4.7H_20$	430	
KI	41.5	
$Na_2MoO_4.2H_2O$	12.5	
$CuS0_4.5H_2O$	1.25	
$CoCl_2.6H_20$	1.25	
VS stock		
Meso-inositol	5000	10
Pyridoxine-HCl	25	
Thiamine-HCl	5	
Nicotinic acid	25	
Glycine	100	

*While preparation of stocks, all chemicals should be dissolved one by on otherwise precipitation may occur

APPENDIX-III

*Stock solution (6mM) of STS

Components	Concentration in mM
$AgNO_3$	12
$Na_2S_2O_3$	96
*components were added in 1: 1 ratio.	