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In Vitro Propagation and Conservation of Stevia Rebaudiana Bertoni

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IN VITRO PROPAGATION AND CONSERVATION OF *STEVIA REBAUDIANA* BERTONI
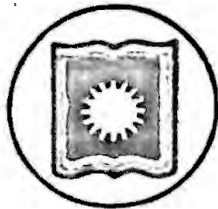
A THESIS SUBMITTED FOR THE DEGREE OF

MASTER OF PHILOSOPHY

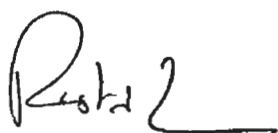IN THE INSTITUTE OF BIOLOGICAL SCIENCES,
UNIVERSITY OF RAJSHAHI, BANGLADESH

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Declaration

I hereby declare that the whole work now submitted as a thesis entitled "*In vitro* **Propagation and Conservation of *Stevia rebaudiana* Bertoni**" in the Institute of Biological Sciences, University of Rajshahi for the degree of Master of Philosophy is the result of my own investigation. The thesis contains no material which has been accepted for the award of any other degree or diploma elsewhere, and to the best of my knowledge, the thesis contains no material previously published or written by another person, except where due reference is made in the text.

(Rashed Zaman)
Candidate

Certificate

This is to certify that M Rashed Zaman worked under my supervision as a M Phil Fellow, I am pleased to forward his thesis entitled *"In vitro* **Propagation and Conservation of Stevia rebaudiana** Bertoni"* which is carried out in the Institute of Biological Sciences, University of Rajshahi. He has fulfilled all the requirements of the regulations and prescribed period of research for submission of thesis for the award of the degree of **Master of Philosophy**.

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ACKNOWLEDGEMENT

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List of Abbreviations

IAA .... 1H indole-3-acetic acid

IBA .... 1H indole-3-butyric acid

NAA .... 1-naphthaleneacetic acid

2,4-D .... 2,4-dichlorophenoxyacetic acid

BAP 6-benzylaminopurine

Kn .... 6-furfurylaminopurine (Kinetin)

MS .... Murashige & Skoog (1962) medium

$HgCl_2$ .... Mercuric chloride

HCl .... Hydrochloric acid

KOH .... Potassium hydroxide

NaOH .... Sodium hydroxide

0.1N .... 0.1 Normal solution

pH .... Negative logarithm of hydrogen ion concentration

°C .... Degree centigrade

No. .... Number

Fig. .... Figure

cm .... Centimetre (s)

g .... Gram (s)

mg .... Milligram

l .... Litre

ml .... Mililitre

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ABSTRACT

Experiments were conducted on *in vitro* culture of *Stevia rebaudiana* Bertoni, an important non-caloric sweetening herb to explore its potential for micro-propagation. Nodes, internodes and leaves of pot grown mature plants were used as explants and experiments were based on surface sterilization; direct shoot proliferation; callus formation; indirect shoot proliferation from callus and root proliferation of induced shoot.

Normal process of tissue culture technique was followed for *in vitro* culture of the different explants. Different concentration of Auxins (IAA, IBA, NAA, 2,4-D) and Cytokinins (BAP, Kn) were used individually or in combinations in the culture media as growth regulator supplements. For surface sterilization, 2 minutes of Mercuric chloride ($HgCl_2$) treatment was found the most effective for leaves and 2.5 minutes for nodes and internodes.

Nodes showed the highest percentage of direct shoot proliferation compared to internodes. BAP (2.0 mg/l) + NAA (0.2 mg/l) was the best combination for direct shoot proliferation. When this combination of growth regulators was used in the culture media, highest percentage of proliferated shoots from nodes was 92.84 ± 0.45, average number of shoots per explant was 4.16 ± 0.28 and average length of the longest shoot was 2.67± 0.98 cm. In same combination, highest percentage of proliferated shoots from internodes was 84.87 ± 0.62, average number of shoots per explant was 3.95 ± 0.34 and average length of the longest shoot was 2.78 ± 0.43 cm.

But internodes showed the highest percentage of callus formation compared to nodes and leaves. 2,4-D (2.5 mg/l) + NAA (1.5 mg/l) was the best combination for callus

formation. When this combination of growth regulators was used in the culture media, highest percentage of callus formation was 82.69 ± 1.40 from the internodes, average day of callus initiation was 10.00 ± 0.05 and colour of callus was green. In case of nodes, highest percentage of callus formation was 70.34 ± 0.39, average day of callus initiation was 10.50 ± 0.15 and colour of callus was green. Again in same combination highest percentage of callus formation was 68.21 ± 0.87 when leaves were used as explants, average day of callus initiation was 11.00 ± 0.15 and colour of callus was also green.

Callus of nodes showed the highest percentage of indirect shoot proliferation compared to callus of internodes and leaves. BAP (2.0 mg/l) + NAA (0.2 mg/l) was the best combination for direct shoot proliferation. When this combination of growth regulators was used in the culture media, highest percentage of proliferated shoots from callus of the nodes which was 94.70 ± 0.72, average number of shoots per callus was 4.25 ± 0.30 and average length of the longest shoot was 2.58 ± 0.12 cm. In same combination highest percentage of proliferated shoots from callus the internodes was 89.15 ± 1.05, average number of shoots per callus was 4.20 ± 0.16 and average length of the longest shoot was 2.73 ± 0.24 cm. Again highest percentage of proliferated shoots from callus of the leaves was 84.73 ± 1.17 in same combination, average number of shoots per callus was 3.67 ± 0.42 and average length of the longest shoot was 2.57 ± 0.36 cm.

Induced shoots from nodes showed the highest percentage of root proliferation compared to induced shoots from internodes and leaves. NAA (0.2 mg/l) was the best concentration for root proliferation. When this concentration of NAA was used in the culture media, highest percentage of proliferated roots was recorded 93.33 ± 0.48 from induced shoots of the nodes, average number of roots per shoot was 10.40 ± 0.62 and average length of the longest root was 2.30 ± 0.15 cm. Highest percentage of proliferated roots from induced shoots of the internodes was 92.45 ± 0.72, average number of roots per shoot was 9.08 ± 0.28 and average length of the longest root was 2.38 ± 0.08 cm. Again in same concentration of NAA highest percentage of proliferated roots was recorded 92.21 ± 0.33

from induced shoots of the leaves, average number of roots per shoot was 10.23 ± 0.55 and average length of the longest root was 2.36 ± 0.13 cm.

After acclimatization percentages of survived plantlets from nodes, internodes and leaves were recorded 82.64%, 81.38%, and 76.53% respectively after 4th week of transplantation.

In the present investigation, *in vitro* propagation of *Stevia* has been demonstrated with its overall potentiality and suitability. *In vitro* propagation can become an important alternative to conventional propagation and breeding procedures for wide range of plant species.
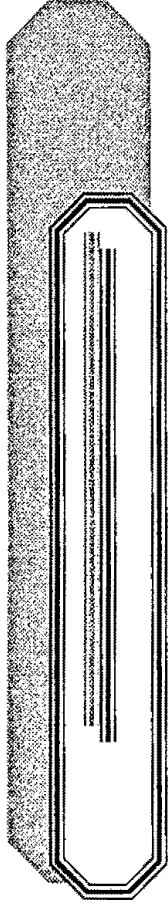
Chapter 1

Introduction

Chapter 1

INTRODUCTION

1.1   Introduction

Plants are an important source of food and medicines and play a key role in world health (Constabel 1990). Almost all cultures from ancient times to today have used plants as medicine. Today medicinal plants are important to the global economy (Srivastava *et al.* 1995), as approximately 85% of traditional medicine preparations involve the use of plants or plant extracts (Vieira and Skorupa 1993). In the past few decades there has been a resurgence of interest in the study and use of medicinal plants in health care and in recognition of the importance of medicinal plants to the health system (Lewington 1993, Mendelsohn and Balick 1994, Hoareau and DaSilva 1999). This awakening has led to a sudden rise in demand for herbal medicines, followed by a belated growth in international awareness about the dwindling supply of the world's medicinal plants (Bodeker 2002). Most of the pharmaceutical industry is highly dependent on wild populations for the supply of raw materials for extraction of medicinally important compounds. The genetic diversity of medicinal plants in the world is getting endangered at an alarming rate because of ruinous harvesting practices and over-harvesting for production of medicines, with little or no regard to the future. Also, extensive destruction of the plant-rich habitat as a result of forest degradation, agricultural encroachment, urbanization, etc. are other factors. Hence there is a strong need for proactive understanding in the conservation, cultivation, and sustainable usage of important medicinal plant species for future use.

In modern medicine, plants are used as sources of direct therapeutic agents, as models for new synthetic compounds, and as a taxonomic marker for discovery of new

compounds. They serve as a raw material base for the elaboration of more complex semisynthetic chemical compounds (Akerele 1992). The synthesis of bioactive compounds chemically is difficult because of their complex structure and high cost (Shimomura et al. 1997). Wide variations in medicinal quality and content in phytopharmaceutical preparations have been observed. These are influenced mainly by cultivation period, season of collection, plant-to-plant variability in the medicinal content, adulteration of medicinal preparations with misidentified plant species, a lack of adequate methods for the production and standardization of the crop, a lack of understanding of the unique plant physiology or efficacy with human consumption, and consumer fraud. Generally, herbal preparations are produced from field-grown plants and are susceptible to infestation by bacteria, fungi, and insects that can alter the medicinal content of the preparations (Murch et al. 2000). It is difficult to ensure the quality control as the medicinal preparations are multi-herb preparations and it is difficult to identify and quantify the active constituents (Wen 2000). Also, there is significant evidence to show that the supply of plants for traditional medicines is failing to satisfy the demand (Cunningham 1993). An efficient and most suited alternative solution to the problems faced by the phytopharmaceutical industry is development of in vitro systems for the production of medicinal plants and their extracts. The in vitro propagated medicinal plants furnish a ready source of uniform, sterile, and compatible plant material for biochemical characterization and identification of active constituents (Wakhlu and Bajwa 1986, Miura et al. 1987). In addition, compounds from tissue cultures may be more easily purified because of simple extraction procedures and absence of significant amounts of pigments, thus possibly reducing the production and processing costs (Chang et al. 1992, 1994).

Plant tissue culture techniques have been increasingly applied to many medicinal plants in particular for mass propagation, conservation of germplasm, study and production of bioactive compounds, and for genetic improvement. Medicinal plants have vast genetic diversity, which is a valuable source of agronomic gene/s of interest for the future. Large-

scale plant tissue culture is found to be an attractive alternative approach to the traditional methods of plantations, as it offers a controlled supply of biochemicals independent of plant availability and more consistent product quality (Sajc et al. 2000). Minimal growth of tissue in culture and cryopreservation have been used to store plant materials from a wide variety of species (Withers 1987). Combinations of in vitro propagation techniques (Fay 1992) and cryopreservation may help in the conservation of biodiversity of locally used medicinal plants. Cryopreservation is a reliable method for long-term storage of the germplasm of endangered species (Bramwell 1990). Several medicinal plant species have been successfully cryopreserved (Bajaj 1995, Naik 1998). Therefore, the principal objective of our research programs was to standardize the protocols of in vitro propagation for the important Chinese medicinal plants.

1.2   *In vitro* propagation

In vitro propagation refers to the true-to-type propagation of selected genotypes using *in vitro* culture techniques. It is an alternative method of propagation (George and Sherrington 1984) and is being used widely for the commercial propagation of a large number of plant species, including many medicinal plants. *In vitro* propagation has been achieved in several medicinal plants using tissue culture techniques (Rout et al. 2000, Nalawade et al. 2003).

Significant progress has been made in the *in vitro* regeneration systems of many traditional Chinese medicinal plants. Using tissue culture protocols for the propagation of superior and/or endangered genotypes of medicinal plants, it is possible to produce healthy and disease-free plants which could be released to their natural habitat or cultivated on a large scale for the pharmaceutical product of interest. These are novel methods of conserving the natural populations of medicinal plants, reducing the risk of their extinction. In vitro propagation techniques impart vigor for the conservation process of the medicinal plants and also maintain the clonal uniformity not achieved by using seeds. The methods could also be used for gene manipulation for crop improvement or more specifically to alter

the expression of gene/s important for the biosynthesis of bioactive compounds. Various strategies for using in vitro systems are being studied extensively with the objective of improving the production and qualitative consistency of plant chemicals. Due to these advances, research in the area of tissue culture technology for production of plant chemicals has bloomed beyond expectations.

Various factors are responsible for *in vitro* morphogenesis. The source of the explant cultured is important in determining the morphogenetic and regenerative potential, which are significantly influenced by the phytosanitary and physiological conditions of the donor plant (Debergh and Maene 1981, Read 1988). Prior to the establishment of aseptic culture, meticulous selection, identification, and maintenance of stock plants used as the source of explants is necessary. Maintaining the donor plants in clean and controlled environmental conditions delivers healthy and sterile explants (Sagare *et al.* 2001). The physiological age of the explants, and the explant type and size are the other factors which exercise an influence on formation of organs in vitro (Rout *et al.* 2000). Temperature, photoperiod, light intensity, pH of medium, carbohydrate source, type of gelling agent, plant growth regulator concentrations in the medium, and additional media amendments also play a determining role in the morphogenesis (Narayanaswamy 1977). The success of any tissue culture protocol depends on the efficient acclimatization of in vitro-obtained plantlets to greenhouse and field conditions. Plantlets growing under *in vitro* conditions exhibit no or reduced photosynthetic capacity, and during acclimatization there is a need for rapid transition from the heterotrophic to the photoautotrophic state for survival (Preece and Sutter 1991). Thus, for effective acclimatization and better adaptation, the in vitro-raised plantlets are gradually exposed to field conditions. Based on the plant species and culture conditions, *in vitro* propagation could be achieved by direct and/or indirect shoot organogenesis and/or somatic embryogenesis.

It is now evident that plant tissue culture is an essential component of Plant Biotechnology which offers novel approaches to the production, propagation, conservation

and manipulation of plants (Thorpe 1993). The success of in vitro culture depends mainly on the growth conditions of the source material (Caswell *et al.* 2000, Delporte *et al.* 2001), medium composition and culture conditions (Sharan *et al* 2004) and on the genotypes of donor plants. How rapidly a tissue grows and the extent and the quality of morphogenetic responses are strongly influenced by the type and concentrations of nutrients supplied (Niedz and Evens 2007). Inorganic macronutrient and micronutrient levels used in most plant tissue culture studies are based on levels established by Murashige and Skoog (1962) for tobacco tissue culture. However, many plant species and varieties do not respond well to the classical approach, i.e. using the MS as the basic medium. This demonstrates that alterations in hormonal ratios cannot be the sole mechanism controlling in vitro developmental processes (Ramage and Willams 2002).

1.3   The Asteraceae (Compositae) family

Asteraceae is one of the large families containing about one-tenth of the total number of flowering plants, comprising about 950 genera and more than 20,000 recognized species and characterized by small flowers arranged in a head looking like a single flower (Rendle 1979).

1.4   The genus *Stevia*

Stevia, one of the 950 genera of the Asteraceae family is a genus of more than 200 species (Gentry 1996). Members of *Stevia* comprise mostly of herbs but also shrubs and trees. Originally it is said to be native to subtropical South America (Paraguay and Brazil) and Central America but now is found over a wide range of areas 500-3500 m altitude, 1,500 – 1,800 mm rain fall and -6 °C to +43 °C temperatures (De Oliveira *et al.* 2004, Midmore and Rank 2002, Yao *et al.* 1999).

Stevia rebaudiana Bertoni

Stevia rebaudiana Bertoni usually grow in semi-dry mountainous terrains, their habitats range from grasslands, forested mountain slopes, conifer forests, to sub-alpine vegetation. It is an herb of 80 - 180 cm tall with a life span of 3 - 5 years. It grows best in soil that is well drained but with reasonable water holding capacity and preferably with pH 5-7; alkaline soil should be avoided (Uddin *et al.* 2006, Midmore and Rank 2002).

For centuries, the Guarani Indian's in Paraguay and Brazil used *Stevia* species, Primarily *S. rebaudiana*, as a non-calorie sweetener in medicinal green teas for treating heart burn and other ailments (Vanek *et al.* 2001); which is 250-300 times sweeter than sucrose at 0.4% solution (Wood *et al.* 1955, Ishima and Katyama 1976, Tanaka 1984, Kim and Kinghorn 2002). Although there are more than 200 species of the genus *Stevia*, only *S. rebaudiana* gives the sweetest essence (Savita *et al.* 2004).

The worldwide demand for high potency sweeteners is expected to increase especially with the new practice of blending different sweeteners, the demand for alternatives is expected to increase. The sweet herb, *S. rebaudiana* produces, in its leaves, just such an alternative with the added advantage that stevia sweeteners are natural plant products. In addition, the sweet steviol glycosides have functional and sensory properties superior to those of many other high potency sweeteners. *Stevia* is likely to become a major source of high potency sweetener for the growing natural food market in the future. The task at hand is to convert stevia from a wild plant to a modern crop well suited to efficient mechanized production. For Canada, the necessary steps are the development of seed, seedling and crop production system, including information on optimized crop inputs, weed and disease control, harvest and handling methods and a breeding program aimed at optimizing glycoside content and sensory characteristics. Understanding the biology of the stevia plants and the chemistry and biochemistry of the sweet glycosides are prerequisites for conversion of stevia to a modern crop.

Now a day it has been used as a natural sweetener substituting sugar, which has no side effects and available as concentrated liquid, crushed leaf or concentrated white powder (Handro and Ferreira 1989).The sweet compound passes through the digestive process without chemically breaking down, making Stevia safe for consumption for those who need to control their blood sugar level. The first report of commercial cultivation in Paraguay was in 1964 (Katayma *et al.* 1976, Lewis 1992). Since then, it has been introduced as a crop in a number of countries including Brazil, Korea, Mexico, United States, Indonesia, Tanzania and Canada (Shock 1982, Saxena and Ming 1988, Takayama and Akita 1994, Fors 1995). In Brazil and Paraguay it grows wild. The property of the species that called attention to the plant was the intense sweet taste of the leaves and aqueous extracts. Stevia sweeteners – extracts from the leaves of this herb – are commercially available in Japan, Korea, China, South-East Asia and South America. Recently, stevia extracts have been extensively used as dietary supplements in the USA (Kyoma *et al.* 2003). Other attributes of this natural, high intensity sweetener include non-fermentable, non-discoloring, maintain heat-stability at 100°C and features a lengthy shelf life. The product can be added to tea and coffee, cooked or baked goods, processed foods and beverages. In the Pacific Rim countries like China, Korea and Japan stevia is regularly used in preparation of food and pharmaceutical products. In Japan alone, an estimated 50 tons of stevioside is used annually with sales valued in order of $220 million Canadian (Brandle and Rosa 1992). It is used as a table top sweetener, in soft drinks, baked goods, pickles, fruit juices, tobacco products, confectionary goods, jams and jellies, candies, yogurts, pastries, chewing gum and sherbets. Stevioside is of special interest to diabetic persons with hyperglycemia and the diet conscious.

Its medicinal and commercial value lead to the world wide demand for large-scale production of stevia plants from elite germplasm. The plant is propagated by seed or stem cutting. Although seed propagation is very common method, seed is not efficient because of low fertility and self incompatibility of the flowers (Felippe and Lucas 1971, Tadhani *et al*

.2006). The propagation by seeds does not allow the production of homogenous population resulting in variability in sweetener level and composition (Nakamura and Tamura 1985, Miyagawa et al.1986). Vegetative propagation by stem cuttings is also limited by the low number of individuals that can be obtained simultaneously from single plant. Micropropagation can provide genetically uniform plants in large numbers. There are few reports of micropropagation from shoot tip, leaf and nodal cultures (Tamura et al. 1984, Ferreira and Handro 1988a, Patil et al. 1996, Sivaram and Mukundan 2003, Mitra and Pal 2007).

1.4.1   Biology, ethnobotany and history of cultivation

Stevia rebaudiana Bert. is one of 154 members of the genus Stevia and one of only two that produce sweet steviol glycosides (Robinson 1930, Soejarto et al. 1982, 1983). It is native to the valley of the Rio Monday in highlands of Paraguay, between 25 and 26 degrees south latitude, where it grows in sandy soils near streams (Katayama et al. 1976). Stevia was first brought to the attention of Europeans in 1887 when M.S. Bertoni learned of its unique properties from the Paraguayan Indians and Mestizos (Lewis 1992). Various reports cited by Lewis (1992) indicate that it was long known to the Guarani Indians of the Paraguayan highlands who called it caá-êhê, meaning sweet herb. The leaves were used either to sweeten maté or as a general sweetening agent. Seeds were sent to England in 1942 in an unsuccessful attempt to establish production. The first reports of commercial cultivation in Paraguay were in 1964 (Katayama et al. 1976, Lewis 1992). A large effort aimed at establishing stevia as a crop in Japan was begun by Sumida (1968). Since then, stevia has been introduced as a crop in a number of countries including Brazil, Korea, Mexico, United States, Indonesia, Tanzania, and, since 1990 Canada (Lee et al. 1979, Donalisio et al. 1982, Goenadi 1983, Schock 1982, Saxena and Ming 1988, Brandle and Rosa 1992, Fors 1995). Stevia production after eighties is centered in China and the major market is in Japan (Kinghorn and Soejarto 1985). No large scale mechanized production has been established and stevia sweeteners are not yet found in mainstream food products

in most countries of the world. Progress towards large scale commercialization has been slow, largely due to difficulties in producing the crop, the poor quality of stevia extracts and the absence of regulatory approvals essential for stevia sweeteners in the North American and European markets.

Stevia is a member of the Compositae family. It is a small shrubby perennial growing up to 65 cm tall, with sessile, oppositely arranged lanceolate to oblancoelate leaves, serrated above the middle. Trichome structures on the leaf surface are of two distinct sizes, one large (4-5 μm), one small (2.5 μm) (Shaffert and Chetobar 1994b). The flowers are small (7-15 mm), white and arranged in an irregular cyme. The seed is an achene with a feathery pappus (Robinson 1930).

Stevia is an obligate short day plant with a critical day length of about 13 h. Extensive variability within populations for day length sensitivity has been reported (Valio and Rocha 1966, Zaidan *et al.* 1980). Plants can initiate flowering after a minimum of four true leaves have been produced (Carneiro 1990). Sumida (1968) reported the results from a complete diallel cross with 8 parents and found that the amount of selfing ranged between 0 and 0.5%, while outcrossing ranged from 0.7 to 68.7%, indicating that some form of self-incompatibility system is operating (Katayama *et al.* 1976). The reproductive anatomy of the male and female gametophytes is typical for angiosperms (Shaffert and Chetobar 1992, 1994a). *Stevia* is diploid and has 11 chromosome pairs, which is characteristic for most of the South American members of the genus (Frederico *et al.* 1996).

1.4.2 Seed production and quality

Stevia plants can be propagated from cuttings or seed. Since germination rates are poor and seedlings very slow to establish it is best grown as an annual or perrenial transplanted crop. Clonal propagation is practical for small scale production, but is probably not economically viable for large scale production where labor costs are high.

Given stevia's day length requirements, seed production in the Northern hemisphere would be best situated between 20 and 30E N latitude. The crop could be transplanted in February or March and seed collected in late summer. Flowering under these conditions should occur between 54-104 d following transplanting, depending on the daylength sensitivity of the cultivars used for seed production (Katayama et al. 1976). One-thousand seed weights for *Stevia* seed usually range between 0.15 and 0.30 g and, depending on plant density, seed yields of up to 8.1 kg ha^{-1} are possible (Carniero 1990). Seed germination is often poor and rates less than 50% are common (Miyazaki and Wantenabe 1974). Given the aforementioned conditions, seed produced on one ha could be enough to supply transplants for up to 200 ha of leaf production. Seed viability and yield are affected by growing conditions during pollination and seed filling. Excessive rainfall during pollination can affect both seed yield and germination (Carneiro 1990, Shuping and Shizhen 1995). Seed is best stored at 0°C, but even under low temperature conditions germination will still decline 50% over three years (Shuping and Shizhen 1995). Sealing of storage containers or using lower temperatures did not prevent the decrease in germination over time.

1.4.3   Cultural practices

Planting densities ranging from 40,000 to 400,000 plants/ha have been tried in experiments conducted in Japan (Katayama et al. 1976). Leaf yield increased with increasing density up to 83,000 and 111,000 plants ha^{-1} for the first year of production. The concentration of stevioside in the leaves of *Stevia* increases when the plants are grown under long days (Metvier and Viana 1979). Since glycoside synthesis is reduced at or just before flowering, delaying flowering with long days allows more time for glycoside accumulation. It follows that *Stevia* production would be best situated in a long day environment where vegetative period is longer and steviol glycoside yields will be higher.

Fertility requirements for *Stevia* grown as an annual crop are moderate. Results from Japan demonstrate that, at the point of maximum dry matter accumulation, *Stevia*

plants consist of 1.4% N, 0.3% P, and 2.4% K (Katayama *et al.* 1976). Total biomass production of 7500 kg ha $^{-1}$ are possible and of that total, 26% would be roots, 35% stems, and 39% leaves based on the composition observed by Katayama *et al.* (1976) which would require approximately 105 kg N, 23 kg P and 180 kg K from both soil and fertilizer. The actual rates of application will vary according to soil type and production environment, and need to be optimized for each specific situation.

Stevia is harvested just prior to flowering when steviol glycoside content in the leaves is at its maximum (Sumida 1980, Xiang 1983). Following harvest the whole plant is dried and the leaves separated from the stems for further processing (Murai 1988). The stems have very low concentrations of sweet glycosides and are removed to minimize processing costs (Brandle and Rosa 1992). Drying *Stevia* under artificial conditions is affected by a number of factors including loading rate, temperature, and ambient air conditions (Van Hooren and Lester 1992).

1.4.4   Cultivar development

A variety of plant breeding procedures have been used to improve leaf yield and rebaudioside A concentration in the leaves. Based on cultivar descriptions from Japan, China and Korea, it appears that sufficient genetic variability exists to make significant genetic gains in leaf yield, rebaudioside A content and the ratio of rebaudioside-A to stevioside (Brandle and Rosa 1992, Lee *et al.* 1979,1982, Shizhen 1995, Shyu *et al.* 1994, Morita 1987). Brandle and Rosa (1992) found that the heritability of stevioside content to be high (83%), based on calculations from a group of half-sib families. Heritabilities for leaf yield (75 %) and leaf to stem ratio (83 %) were also substantial indicating that selection would be effective. Total sweet glycoside concentration in some lines from China was reported to be as high as 20.5%, and a rebaudioside-A to stevioside ratio of 9:1 was disclosed in the Japanese patent literature (Shizhen 1995, Morita 1987). Two breeding methods reported by the latter authors were: phenotypic mass selection and, recurrent selection for phenotype where selected plants are intercrossed before another round of

selection. Some cultivars such as the high rebaudioside-A selection from Japan, and Suweon 2 and 11 from Korea are based on the selection of single plant and because of self-incompatibility they can only be reproduced vegetatively, which limits their utility.

Nakamura and Tamura (1985) studied a population of 300 random individuals and found that total glycoside concentrations at the seedling and harvest stages were not correlated suggesting that early selection for total glycosides would not be effective. However, the proportion of individual glycosides relative to the total was correlated between seedlings and mature plants making early selection for glycoside composition possible. The authors also observed a wide range of variation in the four main glycosides and found that dulcoside A and stevioside, and rebaudioside A and C, were positively correlated with each other. Stevioside and rebaudioside A, and dulcoside and rebaudioside C, were negatively correlated with each other. These correlations can be partially explained by the biosynthetic relationships between the individual glycosides because stevioside is the substrate for the synthesis of rebaudioside A, plants high in rebaudioside A will probably be low in stevioside (Shibata *et al.* 1991).

1.5   The chemistry of the diterpene glycoside sweeteners

The sweet diterpene glycosides of stevia have been the subject of a number of reviews (Kinghorn and Soejarto 1985, Crammer and Ikan 1986, Hanson and De Oliveira 1993). Although interest in the chemistry of the sweet principles dates from very early in the century, significant progress towards chemical characterization was not made until 1931, with the isolation of stevioside (Bridel and Lavieille 1931a). Treatment of this substance with the digestive juice of a snail yielded three moles of glucose and one mole of steviol, while acid hydrolysis gave isosteviol (Bridel and Lavieille 1931b). Isosteviol was also obtained when steviol was heated in dilute sulfuric acid. Subsequent studies have led to the isolation of seven other sweet glycosides of steviol. Typical proportions, on a dry weight basis, for the four major glycosides found in the leaves of wild stevia plants is 0.3 % dulcoside, 0.6% rebaudioside C, 3.8 % rebaudioside A and 9.1 % stevioside.

1.5.1 Structure of steviol, isosteviol and stevioside

Over 20 years after the pioneering work of Bridel and Lavieille (1931a,b) the structure, stereochemistry and absolute configuration of steviol and isosteviol were established, through a series of chemical reactions and correlations by (Mosettig and Nes 1955, Dolder *et al.* 1960, Djerassi *et al.* 1961, Mosettig *et al.* 1963). Concurrent studies on the parent glycoside indicated that one D-glucopyranose residue, hydrolyzed under alkaline conditions yielding steviolbioside, was attached to a carboxyl group (Wood *et al.* 1955) while the other two were components of a sophorosyl group (Vis and Fletcher 1956) bound to the aglycone through a $-glycosidic linkage (Yamasaki *et al.* 1976). Support for the proposed stereochemistry was achieved by the synthetic transformation of steviol into stevioside (Ogawa *et al.* 1980). Earlier, several approaches to the *in vitro* synthesis of steviol had been reported (Cook and Knox 1970, Nakahara *et al.* 1971, Mori *et al.* 1972, Ziegler and Kloek 1977). Spectroscopic data concerning stevioside and steviolbioside were also published (Van Calsteren *et al.* 1993).

Ibrahim *et al.* (2007) isolated five labdane diterpenoids, austroinulin, isoaustroinulin, sterebin E, sterebin E acetate, and sterebin A acetate, along with hydrocarbons, aliphatic alcohols, β-amyrin, β-sitosterol and stigmasterol from the chloroform soluble fraction of the methanol extract of *Stevia rebaudiana* leaves.

1.5.2 Other diterpenoid glycosides

Further investigation of extracts of *S. rebaudiana* leaves resulted in the isolation and identification of seven other sweet diterpenoid glycosides. The leaves of *S. rebaudiana* are the sources of diterpene glycosides, such as steviolbioside, rubsoside, rebaudioside A, B, C, D, E and F, (Table 1, Fig. 1) dulcoside and stevioside (Starratt *et al.* 2002). Kohda *et al.* (1976) obtained the first two of these, rebaudiosides A and B, from methanol extracts together with the major sweet substance stevioside and steviolbioside, a minor constituent which was first prepared from stevioside by alkaline hydrolysis (Wood *et al.* 1955,

Mantovaneli *et al.* 2004). Subsequently, it was suggested that rebaudioside B was an artifact formed from rebaudioside A during the isolation (Kaneda *et al.* 1977, Sakamoto *et al.* 1977b). Stevioside has been converted by enzymatic and chemical procedures to rebaudioside A (Kaneda *et al.* 1977). Further fractionation of leaf extracts led to the isolation and identification, which was aided by ^{13}C NMR spectroscopy, of three other new sweet glycosides named rebaudioside C, D and E (Sakamoto *et al.* 1977a,b,). Both rebaudioside A and rebaudioside D could be converted to rebaudioside B by alkaline hydrolysis showing that only the ester functionality differed (Kohda *et al.* 1976; Sakamoto *et al.* 1977b). Dulcosides A and B, the latter having the same structure as rebaudioside C, were reported by another laboratory (Kobayashi *et al.* 1977). Labdane diterpene (E.g. sterebins I-N), triterpenes, sterols and flavonoids are some of the non-sweet secondary metabolites that have also been identified from the leaves of *Stevia rebaudiana* (Anonymous 1999, McGarvey *et al.* 2003, Markivic *et al.* 2008).

Table 1. Chemical constituents of *Stevia rebaudiana* Bertoni

Compounds Name	R_1	R_2
Stevioside	β-Glc	β-Glc-β-Glc(2→1)
Steviolbioside	H	β-Glc-β-Glc(2→1)
Rebaudioside A	β-Glc	β-Glc-β-Glc(2→1) | β-Glc(3→1)
Rebaudioside B	H	β-Glc-β-Glc(2→1) | β-Glc(3→1)
Rebaudioside C	β-Glc	β-Glc-α-Rha(2→1) | β-Glc(3→1)
Rebaudioside D	β-Glc-β-Glc(2→1)	β-Glc-β-Glc(2→1) | β-Glc(3→1)
Rebaudioside E	β-Glc-β-Glc(2→1)	β-Glc-β-Glc(2→1)
Rebaudioside F	β-Glc	β-Glc-β-Xyl(2→1) | β-Glc(3→1)
Ducloside A	β-Glc	β-Glc-α-Rha(2→1)
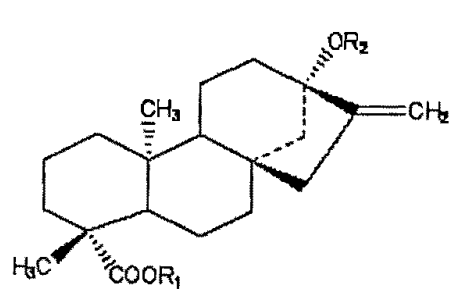
Fig. 1. Structures of diterpene glycosides

1.5.3   Other constituents

In addition to the sweet diterpenoid glycosides, several other diterpenes have been isolated from stevia. Since these compounds may be part of the waste stream produced during stevia processing, their availability in large quantities could make them into valuble co-products. The first to be characterized were jhanol and austroinulin, previously obtained from other plants, and 6-O-acetylaustroinulin (Sholichin et al. 1980). Also reported were the triterpenes $-amyrin acetate and three esters of lupeol and the sterols stigmasterol and $-sitosterol, previously isolated from leaves by Nabeta et al. (1976). Jhanol, austroinulin, 6-O-acetylaustroinulin and 7-O-acetylaustroinulin as well as stevioside and rebaudioside A have been obtained from stevia flowers (Darise et al. 1983). Eight additional diterpenes, called sterebins A-H, have been isolated from leaves and identified (Oshima et al. 1986, 1988).

Other chemical constituents of stevia have been reported. Rajbhandari and Roberts (1983) identified six flavonoid glycosides in an aqueous methanol extract of leaves: apigenin-4'-O-glucoside, luteolin-7-O-glucoside, kaempferol-3-O-rhamnoside, quercitrin, quercetin-3-O-glucoside and quercetin-3-O-arabinoside and 5, 7, 3'-trihydroxy-3, 6, 4'-trimethoxyflavone (centaureidin). The major identified components in the essential oil were the sesquiterpenes $-caryophyllene, trans-$-farnesene, "-humulene, *-cadinene, caryophyllene oxide and nerolidol and the monoterpenes linalool, terpinen-4-ol and "-terpineol (Fujita et al. 1977). Later, Martelli et al. (1985) identified 54 components of a steam distillate of dried leaves from Brazil. Of these, caryophyllene oxide and spathulenol were the main components, totaling 43%. Interestingly, these substances were not the major components in an essential oil preparation from a fresh sample of cultivated stevia plants from Italy.

1.5.4   Functional and sensory properties of steviol glycoside sweeteners

Of the four major sweet diterpene glycoside sweeteners present in stevia leaves only two, stevioside and rebaudioside A, have had their physical and sensory properties

well characterized. Stevioside and rebaudioside A were tested for stability in carbonated beverages and found to be both heat and pH stable (Chang and Cook 1983). However, rebaudioside A was subject to degradation upon long term exposure to sunlight. Kinghorn and Soejarto (1985) also cite numerous Japanese studies that demonstrate that stevioside is very stable.

Phillips (1989) has summarized the early sensory research. Stevioside was between 110 and 270 times sweeter than sucrose, rebaudioside A between 150 and 320, and rebaudioside C between 40 and 60. Dulcoside A was 30 times sweeter than sucrose. Rebaudioside A was the least astringent, the least bitter, had the least persistant aftertaste and was judged to have the most favourable sensory attributes of the four major steviol glycosides (Phillips 1989, Tanaka 1997). Dubois and Stephanson (1984) have also confirmed that rebaudioside A is less bitter than stevioside and demonstrated that the bitter notes in stevioside and rebaudioside A are an inherent property of the compounds and not necessarily the result of impurities in whole plant extracts. Relative to other high potency sweeteners such as apsartame, bitterness tends to increase with concentration for both stevioside and rebaudioside A (Schiffman et al. 1994). Both stevioside and rebaudioside A are synergistic in mixtures with other high potency sweeteners such as aspartame and are good candidates for inclusion in blends (Schiffman et al. 1995). Although specialty applications may exist for the other glycosides, increasing levels of rebaudioside A in stevia leaves is a clear objective for breeding work.

1.5.5   Commercial extraction of steviol glycosides

Most of the commercial processing of stevia leaves occurs in Japan and there are dozens of patents describing methods for the extraction of steviol glycosides. Kinghorn and Soejarto (1985) have categorized the extraction patents into: those based on solvent (Haga et al. 1976), solvent plus a decolorizing agent (Ogawa 1980), adsorption chromatography (Itagaki and Ito 1979), ion exchange (Uneshi et al. 1977), and selective precipitation of individual glycosides (Matsushita and Kitahara 1981). Phillips (1989) has indicated that the

most favoured extraction processes involve four steps: aqueous or solvent extraction, ion exchange, precipitation or coagulation with filtration, then crystallization and drying. New methods based on ultra-filtration have also been disclosed (Tan and Ueki 1994).

1.5.6 Pharmacological activities of *Stevia rebaudiana* Bertoni

S. rebaudiana extracts have been suggested to exert beneficial effects on human health, including antihypertensive (Chang *et al.* 1994, Jeppensen *et al.* 2003), cardiovascular (Haebisch 1992, Melis 1992a), antimicrobial (Tadhani and Subhash 2006, Jayaraman *et al.* 2008, Ghosh *et al.* 2008), anticancerous (Jeppensen *et al.* 2003, Rajesh *et al.* 2010), contraceptive (Melis 1999), antiobesity and antioxidant activities (Park and Cha 2010) and also thought to influence glucose metabolism and renal functions (Melis and Sainati 1991, Melis 1992b, Savita *et al.* 2004, Jeppesen *et al.* 2003), cytoprotective antiulcerous (Pandiyan *et al.* 2009) and prevention of dental caries (Fujita *et al.* 1979). *Stevia* can also inhibit bacteria and fungal growth (Rojas and Miranda 2002). *S. rebaudiana* does not lower blood glucose levels in normal subjects (Ahmed and Smith 2002).

1.6　　Objectives of the study

Cultivation of *S. rebaudiana* in experimental scale has been conducted recently in Bangladesh in attempting to exploit the possibility of using stevioside as a natural substrate for synthetic sweeteners. However, since seeds are usually sterile (Yang *et al.* 1981), small size and their self incompatibility (Midmore and Rank 2002) poor seed germination is the major factor limiting large-scale cultivation of this plant (Goettemoeller and Ching 1999, Lester 1999). Propagation by seeds does not allow the production of homogenous plant population, resulting in great variability in important features like sweetening levels and composition (Tamura *et al.* 1984, Nakamura and Tamura 1985). Vegetative propagation too, is limited by lowering number of individuals that can be obtained from single plant (Sakaguchi and Kan 1982). Other propagation methods such as stem cuttings are used, which easily but requires high labour inputs and thus costly. These tend to be slow and impractical when carried out on a large scale for propagation of selected elite individuals. Due to above mentioned difficulties; tissue culture is the only alternative for rapid mass propagation of *Stevia* plants. Synthetic growth regulators enhance and accelerate the production of *in vitro* plants with good agronomical traits. Tissue culture propagation of this species, therefore, offers a possible alternative. The present investigation was undertaken to find out suitable sources of explants and suitable concentration of 2,4-D for callus induction in micro propagation of *S. rebaudiana*.

(Whole plant with flower)

(Stem with leaves)

Plate 1. *Stevia rebaudiana* plant
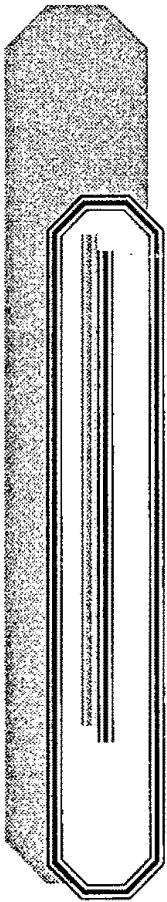
Chapter 2

Materials
and
Methods

MATERIALS AND METHODS

Materials and Methods

This chapter presents the materials and the methods that were used in conducting the investigation.

2.1   Materials

To conduct the present investigation following materials and equipments were used:

2.1.1   Plant Materials

The pot grown *Stevia rebaudiana* plants were collected from the Tissue Culture Laboratory of BARC, Gazipur. Nodes, Internodes and Leaves of healthy *Stevia rebaudiana* plants were used as explants for the investigation.

2.1.2   Surface sterilant and surfactant

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

2.1.3   Chemicals and sources

The chemical compound used as macronutrients and micronutrients in the present study were reagent grade (GPR) products of either Ricdel-de-Haen, Germany; BDH, England/India or E. Merck, Germany/India and Phyto Technology Laboratories ™ USA. The

vitamins and growth regulators were mostly products of Phyto Technology Laboratories [TM] USA/E. Merck, India. A small section of them was procured from BDH, England.

2.1.4   Culture media

The nutrient media used in plant tissue culture are composed of several components, salts, vitamins, amino acids, growth regulator, sugars, agar or gelrite and water. All these compounds full-fill one or more functions in the *in vitro* growth of plants. The excised explants can only grow *in vitro* on a suitable artificially prepared nutrient medium which is known as culture medium. From time to time, many workers/scientists (White, Murashige and Skoog, Gamborg, Nitsch and Nitcsh, Schenk, Hildebrandt and others) have proposed the composition of different nutrient media for *in vitro* growth and development of explant tissues. MS (Murashige and Skoog 1962) medium was used in the present study and chemical composition of the media are listed in Table 2.

Table 2. Chemical composition of MS media (1 litre)

Macronutrients		Micronutrients		Organic supplements	
Chemical	Amount (g)	Chemical	Amount (mg)	Chemical	Amount (mg)
NH_4NO_3	1.65	$FeSO_4.7H_2O$	27.80	Myoinositol	100.00
KNO_3	1.90	$Na_2EDTA.2H_2O$	33.60	Nicotinic acid	0.05
$CaCl_2.2H_2O$	0.44	KI	0.83	Pyridoxine HCl	0.05
$MgSO_4.7H_2O$	0.37	H_3BO_4	6.20	Thiamine HCl	0.05
KH_2PO_4	0.17	$MnSO_4.4H_2O$	22.30	Glycine	0.02
		$ZnSO_4.7H_2O$	8.60	Sucrose	30.00
		$Na_2MoO_4.H_2O$	0.25		
		$CuSO_4.5H_2O$	0.025		
		$CoCl_2.6H_2O$	0.025		

2.1.5   Growth regulators

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

i)   Auxins

Auxins promote cell enlargement and root initiation and following four types of Auxins were used to full-fill the experimental purposes:

1H indole-3-acetic acid (IAA)

1H indole-3-butyric acid (IBA)

1-naphthaleneacetic acid (NAA)

2,4-dichlorophenoxyacetic acid (2,4-D)

ii)   Cytokinins

Cytokinins promote cell division and shoot initiation and following two types of cytokinins were used to full-fill the experimental purposes:

6-benzylaminopurine (BAP)

6-furfurylaminopurine (Kinetin or Kn)

2.1.6   Other materials

Other materials used to conducted the present investigation including:

Growth additives

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

Glass wares

Culture tubes, conical flasks, petridishes of various capacities, measuring cylinder (50 ml, 100 ml, 250 ml, 500 ml, 1000 ml), pipettes (0.1 ml, 0.5 ml, 1.0 ml, 2.0 ml, 5.0 ml, 10.0 ml) and beakers of different capacities.

Instruments

Pipette pump, parafilm, cotton plugs, rubber bands, aluminum foils, cotton, water-proof marker pen, bamboo-papers, tiles, culture-tube rack, trolley, waste-basket and stop-watch.

Electronic instruments

Laboratory tables, micro-wave, magnetic stirrer, hot plate, analytical loading single pan balance with precision of 0.001 g, refrigerator, electric hot air oven range up to 250 ± 2°C, digital pH meter, autoclave preferably horizontal, continuous supply of single and double distilled water.

For aseptic transfer

Laminar airflow cabinet, scissors, scalpel handles with blades, various sizes of forceps, sprit lamp and 70% ethyl alcohol.

For incubation

An aseptic culture room including racks with light arrangements and controlled temperature of 26 ± 1° C maintained with air cooler.

2.2   Methods

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

2.2.1   Preparation of sterilant solution

Mercuric chloride ($HgCl_2$) of 0.1% concentration was used for surface sterilization of plant materials used as explants. To prepare 0.1% solution, 0.1 g of $HgCl_2$ was taken in a 250 ml conical flask and dissolved in 100 ml distilled water. Freshly prepared $HgCl_2$ was used for surface sterilization of the explants and $HgCl_2$ was prepared one hour before use.

2.2.2   Preparation of culture media

MS (Murashige and Skoog, 1962) medium was used for direct shoot proliferation, callus formation, indirect shoot proliferation from callus and root proliferation of induced shoot. Growth regulators were added separately to the media according to requirements. Different constituents of MS including growth regulators of the culture media were separated into stock solutions for ready use during the preparation of culture media. Separate stock solution of MS (I-VIII) and growth regulators (GR) and were prepared as follows:

2.2.2.1   Preparation of stock solutions of MS (I-VIII) basal medium

As different media constituents were required in different concentrations, separate stock solution for the macronutrients (stock solutions I, II and III), micronutrients (stock solutions IV, V and VI) and organic supplements (stock solution VII and VIII) were prepared for ready use.

Stock solution-I (Macronutrients)

At first 150 ml distilled water was taken in a conical flask and accurately weighted NH_4NO_3 (16.50 g), KNO_3 (19.00 g) and KH_2PO_4 (1.70 g) were dissolved and the final

volume was made up to 200 ml by further addition of distilled water. It was then filtered and stored in a glass reagent bottle in the refrigerator.

Stock solution-II (Macronutrients)

3.70 g of $MgSO_4.7H_2O$ was weighted and dissolved in 150 ml of distilled water in a conical flask and the final volume was made up to 200 ml by further addition of distilled water. It was then filtered and stored in a glass reagent bottle in the refrigerator.

Stock solution-III (Macronutrients)

4.40 g of $CaCl_2.2H_2O$ was weighted and dissolved in 150 ml of distilled water in a conical flask and the final volume was made up to 200 ml by further addition of distilled water. It was then filtered and stored in a glass reagent bottle in the refrigerator.

Stock solution-IV (Micronutrients)

Two constituents, $FeSO_4.7H_2O$ (0.556 g) and $Na_2EDTA.2H_2O$ (0.746 g) were weighted and dissolved in 350 ml of distilled water in a conical flask and the final volume was made up to 400 ml by further addition of distilled water. It was then stored in dark glass reagent bottle to avoid photosensitive reaction in the refrigerator.

Stock solution-V (Micronutrients)

$MnSO_4.4H_2O$ (0.446 g), H_3BO_3 (0.124 g) and $ZnSO_4.7H_2O$ (0.172 g) were weighted and in 350 ml of distilled water in a conical flask and the final volume was made up to 400 ml by further addition of distilled water. It was then stored in glass reagent bottle in the refrigerator.

Stock solution-VI (Micronutrients)

KI (0.166 g), $CuSO_4.5H_2O$ (0.005 g), $Na_2MoO_4.2H_2O$ (0.05 g) and $CoCl_2.6H_2O$ (0.005 g) were weighted and dissolved in 150 ml of distilled water in a conical flask and the final volume was made up to 200 ml by further addition of distilled water and stored in a glass reagent bottle in the refrigerator.

Stock solution-VII (Organic supplements)

Two gram of Myoinositol was accurately weighted and dissolved in 150 ml of distilled water in a conical flask and the final volume was made up to 200 ml by further addition of distilled water and stored in a glass reagent bottle in the refrigerator.

Stock solution-VIII (Organic nutrients)

Nicotinic acid (0.25 g), Pyridoxine HCl (0.25 g), Thiamine HCl (0.25 g) and Glycine (1.0 g) were weighted and dissolved in 450 ml of distilled water in a conical flask and the final volume was made up to 500 ml by further addition of distilled water and stored in a glass reagent bottle in the refrigerator.

2.2.2.2   Preparation of the stock solutions of growth regulators

In addition to the inorganic and organic nutrients, is generally necessary to add one or more growth regulators mainly Auxins and Cytokinins to the media to support good growth of tissues and organs (Bhojwani and Razdan 1983). Stock solutions of different growth regulators were prepared separately as shown in Table 3.

Table 3. Plant growth regulators and their appropriate solvents for the preparation of growth regulators stock solutions.

Growth regulators (solutes)	Amount of solutes (mg)	Appropriate solvents	Amount of solvents (ml)	Final volume of the stock solution with distilled water (ml)	Strength of the stock solution (mg/ml)
Auxins					
IAA	10.0	70% KOH	1.0	50.0	0.2
IBA	10.0	0.1N KOH	1.0	50.0	0.2
NAA	10.0	0.1N KOH	1.0	50.0	0.2
2,4-D	10.0	70% Ethanol	1.0	50.0	0.2
Cytokinins					
BAP	10.0	0.1N HCl	1.0	50.0	0.2
Kn	10.0	70% KOH	1.0	50.0	0.2

To prepare the stock solution of any of these growth regulators, 10 mg of powdered growth regulator was taken into a clean test tube and then dissolved in required volume of appropriate solvent. The final volume of the solution was made 10 ml by adding distilled water. The stock solution was then poured into glass reagent bottle and after labeling in a refrigerator at 4-5° C for.

2.2.2.3 Preparation of one litre culture media

The following steps were followed to make one litre of culture media based on the instruction chart given in the book of Bhojwani and Razdan (1983).

Step-I: Assembling of stock solutions of MS medium

Twenty ml of stock solution-I, 20 ml of stock solution-II, 20 ml of stock solution-III, 20 ml of stock solution-IV, 20 ml of stock solution-V, 1 ml of stock solution-VI, 10 ml of stock solution-VII, 1 ml of stock solution-VIII were added in a conical flask containing 500 ml distilled water and mixed well.

Step-II: Addition of growth regulators

Different concentrations of growth regulators were added either individually or in different combinations to the solution of step-I and were mixed thoroughly. Since each stock solution of a growth regulator contained 10.0 mg of the chemical in 50.0 ml stock solution (0.2 mg/l), in addition of 5.0 ml of any stock solution of a growth regulator to prepare one litre of media resulted in 1.0 mg/l concentration of that specific growth regulator. So 0.1 mg/l, 0.2 mg/l, 0.3 mg/l, 0.4 mg/l, and 0.5 mg/l concentration of any growth regulators required addition of 0.5 ml, 1.0 ml, 1.5 ml, 2.0 ml and 2.5 ml amounts of that specific growth regulator stock solution respectively for one litre of medium. Similarly 1.0 mg/l, 1.5 mg/l, 2.0 mg/l, 2.5 mg/l, 3.0 mg/l, 4.0 mg/l and 5.0 mg/l concentration of any growth regulators required addition of 5.0 ml, 7.5 ml, 10.0 ml, 12.5 ml, 15 ml, 20 ml and 2.5 ml amounts of that specific growth regulator stock solution respectively for one litre of medium.

Step-III: Addition of sucrose

Thirty gram of sucrose was added to the solution of step-III and mixed thoroughly by a magnetic stirrer. Then the whole mixture was made up to one litre (1000 ml) with further addition of distilled water.

Step-IV: pH adjustment

The pH of the solution was adjusted to 5.7 using a digital pH meter with the help of 0.1N NaOH or 0.1N HCl whichever required.

Step-V: Addition of agar

The nutrient culture media was gelled with agar. In the present experiment 6 g to 10 g agar (on the basis of its quality) was added to one litre medium. The whole mixture was then gently heated in a microwave oven until the agar was melted completely and made the turbid solution clear. At the time of gentle heating, continuous stirring procedure was applied to the solution till complete dissolution of agar.

Step-VI: Dispensation of media

After heating fixed volume of hot medium was dispensed into culture vessels like test tubes or conical flasks. Then the culture vessels were plugged with non-absorbent cotton plugs or aluminum foils at the mouth of the culture vessels.

Step-VII: Sterilization of media

Finally, the media containing culture vessels were sterilized by autoclaving at 121° C for 20 minutes at 1.1 kg/cm² pressure. In case of flasks the media were allowed to cool as vertically but the test tubes containing media were allowed to cool as slants for shoot proliferation or as vertically for callus formation and root proliferation. Then the media containing vessels were stored in the culture room (not more than five days) for ready to use during aseptic manipulations. Before store, the media containing glass vessels were

marked with different codes with the help of a water-proof marker pen to indicate specific growth regulators supplements.

2.2.3 Culture techniques

Techniques used in the present investigation for the regeneration of complete plantlets from *Stevia rebaudiana* are described under the following headings:

2.2.3.1 Isolation and sterilization of explants

Healthy, disease free and desired plant parts were collected from open environment grown plants and washed thoroughly under running tap water. The plant materials were then brought to laboratory and were thoroughly washed under running tap water and placed in separate flasks. Then the materials were washed 3 to 4 times with distilled water and taken in front of the running laminar air flow cabinet and transferred to 250 ml sterilized conical flask. After rinsing with 80% ethanol for 30 seconds they were immersed in 0.1% $HgCl_2$ for different durations of time. The materials were then washed with autoclaved distilled water with at least three changes to remove all traces of $HgCl_2$.

2.2.3.2 Preparation of explants

The sterilized plant materials were placed on a sterilized petridish and cut into small pieces with a sharp scalpel. Three different explants were used in the present investigation namely,

Nodes: Excised shoots each with a single node of approximately 1.0 cm to 2.0 cm in length.

Internodes: Excised shoots of approximately 1.0 cm to 1.5 cm in length.

Leaves: Tender leaves were excised into small segments.

All the dissections of plant materials were carried out in front of the running laminar air flow cabinet for the preparation of explants and all the dissecting materials were sterilized by autoclave.

2.2.3.3   Inoculation

The excised explants (nodes, internodes, leaves) were cultured singly into 25X150 mm culture tube. Each culture tube contained 15 ml to 20 ml of agar gelled culture media supplemented with individual or combination of growth regulators. All these works were conducted aseptically in front of the running laminar air flow cabinet with the help of a pair of sterile forceps.

2.2.3.4   Incubation

The inoculated tubes were then incubated in an air conditioned culture room which provided special culture environment. The tubes were placed on the shelves of culture-tube racks in the culture room. The cultures were maintained at 26 ± 1° C of temperature with light intensity of 2000-3000 lux (50-70 micro E. m^{-2}) provided by cool-white florescent tubes. Photoperiod was maintained as 16 h of light and 8 h of dark. The culture tubes were checked daily to note the morphogenic response of different experiments conducted in the present investigation.

2.2.3.5   Direct shoot proliferation from the different explants

In the culture room different explants (nodes, internodes) of the culture tubes containing specific growth regulators for shooting showed direct shoot proliferation within few days. After four weeks of inoculation data were recorded carefully to calculate percentage of explants showing shoot proliferation and after six weeks data were recorded again to calculated average number of shoots per explant and average length of the longest shoots. Each treatment consisted of three replications and in each replication 10-15 explants were used. The usable shoots were then excised from the shoot culture and transferred them individually to the culture media containing specific growth regulator for rooting.

2.2.3.6   Callus formation from the different explants

Within few days different explants (nodes, internodes, leaves) of the culture tubes containing specific growth regulators for callus induction showed callus formation in the culture room. Colour of each callus was observed and data were recorded carefully to calculate the average day of callus initiation and percentage of explants induced callus. Each treatment consisted of three replications and in each replication 10-15 explants were used. After attaining a convenient, size they were transferred individually to the culture media containing specific growth regulator for indirect shoot proliferation.

2.2.3.7   Indirect shoot proliferation from callus of the different explants

For indirect shoot proliferation well grown up individual callus of four weeks of age was taken aseptically on a sterile petridish and cut into small and suitable sizes by a sterile scalpel and inoculated into the culture tubes containing culture media with specific growth regulators for shooting. The inoculated tubes were then incubated into the culture room. In the culture room callus of different explants (nodes, internodes, leaves) of the culture tubes containing specific growth regulators for shooting showed indirect shoot proliferation within few days.   After four weeks of inoculation data were recorded carefully to calculate the percentage of callus showing shoot proliferation and after six weeks data were recorded again to calculated average number of shoots per callus and average length of the longest shoots. Each treatment consisted of three replications and in each replication 10-15 explants were used. The usable shoots were then excised from the shoot culture and transferred them individually to the culture media containing specific growth regulator for rooting.

2.2.3.8   Root proliferation from induced shoots of the different explants

For root proliferation, the new induced shoots were separated aseptically from the shoot messes and the individual shoots were then inoculated into the culture tubes containing culture media with specific growth regulators for rooting. The inoculated tubes

were then incubated into the culture room. Within few days inoculated shoots of the culture tubes showed root proliferation in the culture room. After four weeks of inoculation data were recorded carefully to calculate the percentage of shoots showing root proliferation, average number of roots per shoot and average length of the longest roots. Each treatment consisted of three replications and in each replication 10-15 explants were used.

2.2.3.9   Precautions for ensuring aseptic condition

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

2.2.4   Transplantation of plantlets under ex *vitro* environment

After developing sufficient root system the regenerated plantlets were considered ready to transfer in soil. Plantlets with sufficient root system, which were grown inside the culture vessels, were kept unplugged for 4 to 5 days .Then they were brought out from the controlled environment of the culture room and kept is the room temperature for 6 to 10 days to bring them in contact with normal temperature. The plantlets were then transferred carefully from the culture vessels and roots of the plantlets were gently washed under running tap water to remove culture media attached to the root zone. Immediately after that they were transferred to small pots filled with garden soil, organic manure and sand in the portion of 2:2:1, taking special care not to damage of the roots. The pots with the plantlets

were covered with small transparent polythene bags to prevent sudden desiccation. The inner sides of the bags were sprayed with water at every 8 hours to maintain high humidity around the plantlets. The polythene bags were gradually perforated to expose the plantlets to the outer normal environment and subsequently removed after 8 to 10 days. By this time the potted plantlets became established in the soil. This practice facilitated gradual acclimatization of the *in vitro* grown new plantlets in ex *vitro* environment. After resuming the new growth on soil having sufficient light extension and leaf development, the plantlets were transferred to the larger earthen pots filled with organic manure and soil and watered regularly. For the initial period of 30 to 40 days the potted plantlets were nurtured in the laboratory condition under artificial light intensity of 2000-3000 lux (50-70 micro E. m^{-2}) and then shift under open sunlight and they became suitable for final plantation in the fields. Data were recorded carefully after 1st, 2nd, 3rd and 4th week of transplantation to calculate the percentage of survived plantlets.

2.2.5   Calculation and presentation of data

For presentation of data in the tables and figures, statistical methods were used to quantify the experimental results. Percentages, means etc. were calculated by normal mathematical methods and statistical calculations like standard error (SE) of means was performed according to standard statistical procedure (Steel and Torrie 1982).
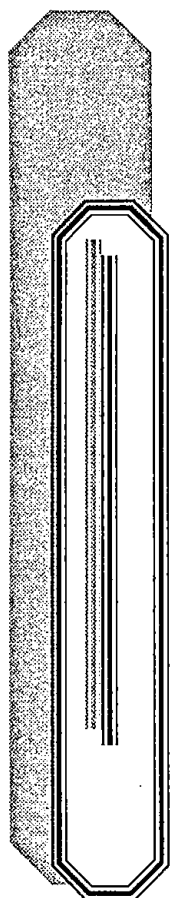
Chapter 3

Results

RESULTS

Investigations were carried out with different explants of *Stevia rebaudiana* Bertoni to standardize suitable protocols for surface sterilization; direct shoot proliferation; high frequency of callus formation; indirect shoot proliferation from the callus and root proliferation of the induced shoots for subsequent plant regeneration and the results of the investigations are described under the following heads:

3.1   Surface sterilization of the different explants

To establish the explants from field grown plants under aseptic condition, surface sterilization is essential. Standardization for surface sterilization was carried out by trial and error experiments with 0.1% Mercuric Chloride ($HgCl_2$) solution at different time duration ranges from 0.5 to 3.0 minutes. The effects $HgCl_2$ treatment with different duration of time on surface sterilization of different the explants (Nodes, Internodes and leaves) are presented in Table 4.

3.1.1   Effects of $HgCl_2$ treatment on nodes

In case of nodes, the ranges of percentage of free contamination was between 10.68 ± 0.83 to 93.22 ± 0.58 and percentage of survived explant was between 10.68 ± 0.83 to 77.48 ± 0.35. Percentage of free contamination was found 93.22 ± 0.58 when $HgCl_2$ treatment was used for 3.0 minutes but percentage of survived explant was 64.51 ± 0.97. Percentage of free contamination was 81.94 ± 0.94 and percentage of survived explants was 77.48 ± 0.35 when the nodes were treated for 2.5 minutes with $HgCl_2$. Again when $HgCl_2$ treatment was used for 2.0 minutes, percentage of free contamination and percentage of survived explant were 67.32 ± 0.86 and 64.40 ± 1.34 respectively. So by

comparing both, the percentage of free contamination and the percentage of survived explant, HgCl$_2$ (0.1%) treatment for 2.5 minutes of duration was found the most effective for the surface sterilization of nodes of *Stevia*.

3.1.2   Effects of HgCl$_2$ treatment on internodes

In case of internodes, the ranges of percentage of free contamination was between 11.35 ± 0.92 to 96.54 ± 1.81 and percentage of survived explant was between 11.35 ± 0.92 to 79.83 ± 0.83. Percentage of free contamination was 96.54 ± 1.81 when the internodes were treated with HgCl$_2$ for 3.0 minutes and percentage of survived explant was 67.25 ± 1.05. But when the internodes were treated with HgCl$_2$ for 2.5 minutes, percentage of free contamination was 84.43 ± 0.58 and percentage of survived explant was 79.83 ± 0.83. Percentage of free contamination was 72.29 ± 0.45 and percentage of survived explant was 68.29 ± 0.97 when the internodes were treated with HgCl$_2$ for 2.0 minutes. So by comparing both, the percentage of free contamination and the percentage of survived explant, HgCl$_2$ (0.1%) treatment for 2.5 minutes of duration was found the most effective for the surface sterilization of internodes of *Stevia*.

3.1.3   Effects of HgCl$_2$ treatment on leaves

In case of leaves the ranges of percentage of free contamination was between 18.20 ± 1.48 to 98.67 ± 0.72 and percentage of survived explant was between 18.20 ± 1.48 to 77.11 ± 1.11. Percentage of free contamination was 98.67 ± 0.72 but percentage of survived explant was only 52.18 ± 0.53 when the leaves were treated with HgCl$_2$ for 3.0 minutes. Again percentage of free contamination was 95.82 ± 0.66 when the leaves were treated with HgCl$_2$ for 2.5 minutes and percentage of survived explant was 76.92 ± 0.76. But when the leaves were treated with HgCl$_2$ for 2.0 minutes, percentage of free contamination was 87.08 ± 1.27 and percentage of survived explant was 77.11 ± 1.11. So by comparing both, the percentage of free contamination and the percentage of survived

explant, $HgCl_2$ (0.1%) treatment for 2.0 minutes of duration was found the most effective for the surface sterilization of Leaves of stevia.

The effects of $HgCl_2$ (0.1%) treatment for different duration of times on nodes, intemodes and leaves are presented by histogram graph in Fig.2.

Table 4. Effects of $HgCl_2$ treatment with different duration of time on surface sterilization of the different explants of *Stevia rebaudiana*.

Time (minutes)	Percentage of free contamination			Percentage of survived explants		
	Nodes	Internodes	Leaves	Nodes	Internodes	Leaves
0.5	10.68 ± 0.83	11.35 ± 0.92	18.20 ± 1.48	10.68 ± 0.83	11.35 ± 0.92	18.20 ± 1.48
1.0	32.55 ± 0.68	34.82 ± 1.26	41.38 ± 0.77	32.55 ± 0.68	34.82 ± 1.26	41.3 ± 0.77
1.5	48.89 ± 0.55	53.46 ± 0.73	58.14 ± 1.39	45.39 ± 0.44	51.08 ± 0.68	54.36 ± 0.40
2.0	67.32 ± 0.86	72.29 ± 0.45	87.08 ± 1.27	64.40 ± 1.34	68.29 ± 0.97	77.11 ± 1.11
2.5	81.94 ± 0.94	84.43 ± 0.58	95.82 ± 0.66	77.48 ± 0.35	79.83 ± 0.83	76.92 ± 0.76
3.0	93.22 ± 0.58	96.54 ± 1.81	98.67 ± 0.72	64.51 ± 0.97	67.25 ± 1.05	52.18 ± 0.53
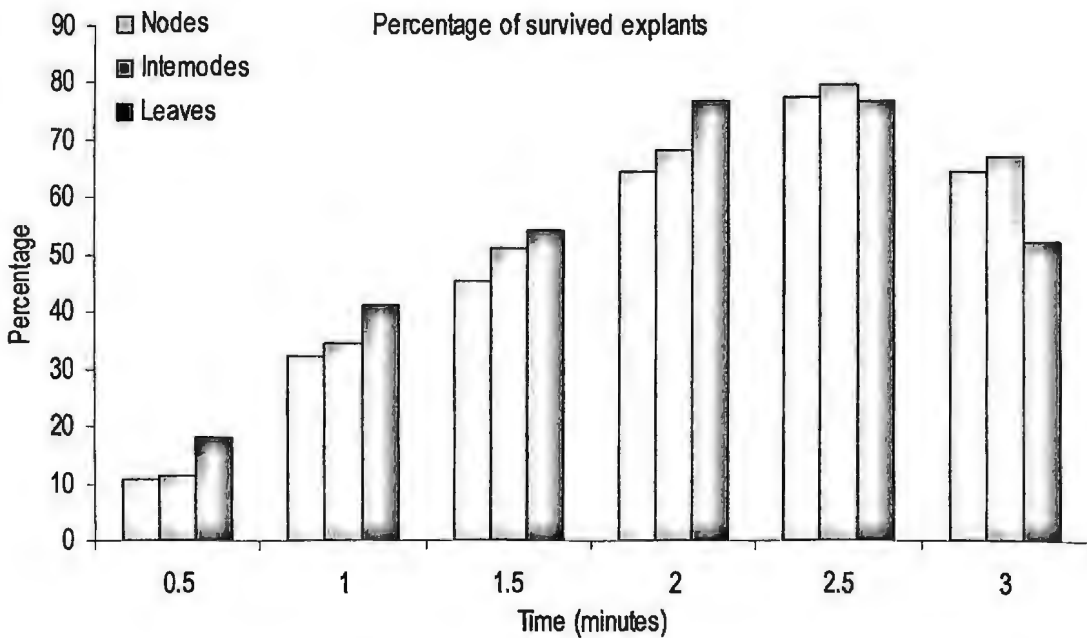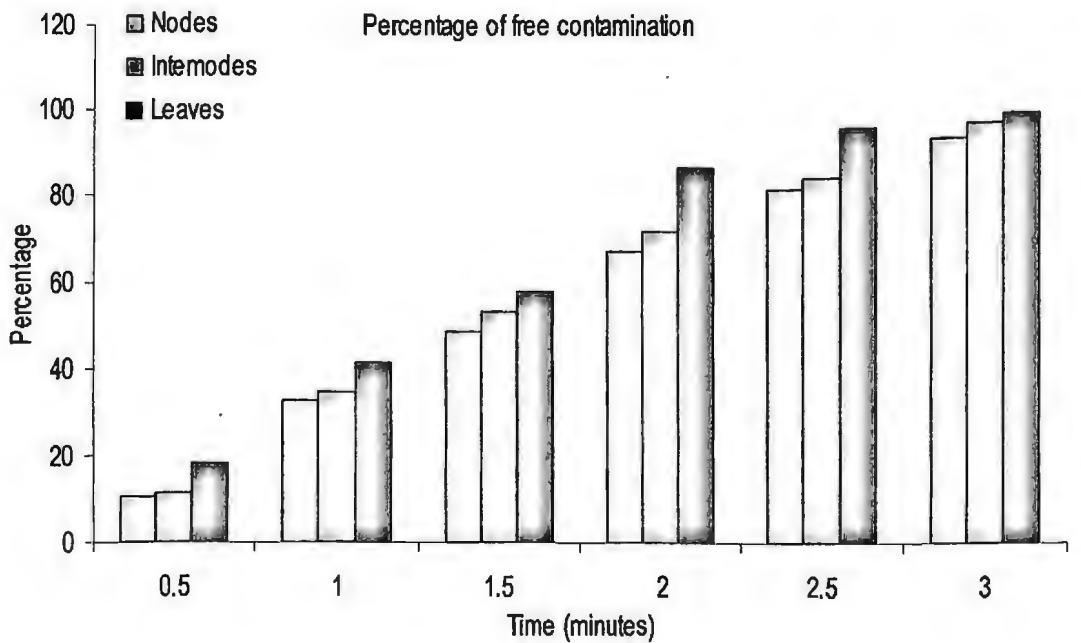
Fig. 2. Effects of HgCl$_2$ (0.1%) treatment for different duration of times on surface sterilization of the different explants of *Stevia rebaudiana*; percentage of free contamination (upper) and percentage of survived explants (lower).

3.2   Direct shoot proliferation from the different explants

Different concentration of Cytokinins and Auxins were used individually and in combination in the culture media to observe the effects of the growth regulators on direct shoot proliferation from nodes, and internodes. In all cases nodes showed the highest percentage of proliferated shoots compared to internodes (Plate 2).

3.2.1   Effects of individual growth regulators on direct shoot proliferation from the different explants

Two types of Cytokinins (BAP and Kn), each with six different concentrations (0.5 mg/l, 1.0 mg/l, 1.5 mg/l, 2.0 mg/l, 2.5 mg/l, 3.0 mg/l) were used in the culture media to find out the effects of individual growth regulators on direct shoot proliferation from nodes and internodes and the results are presented in Table 5. Kn was found less effective than BAP on direct shoot proliferation from the different explants when they were used individually in the culture media.

3.2.1.1   Effects of BAP on direct shoot proliferation

Shoot proliferation rate from nodes and internodes and was average when BAP was used singly in the culture media in different concentration. Nodes showed the highest percentage of proliferated shoots which was 57.67 ± 0.57 in the culture media with BAP (2.0 mg/l). Average number of shoots per explant was 3.62 ± 0.12 and average length of the longest shoot was 2.68 ± 0.21 cm. But in case of internodes, highest percentage of proliferated shoots was 44.49 ± 0.92 in the same concentration of BAP and the average number of shoots per explant was 3.47 ± 0.22 and average length of the longest shoot was 2.32 ± 0.73 cm. Other concentrations of BAP showed average results. The effects of different concentration of BAP on direct shoot proliferation from the different explants are presented by histogram graph in Fig. 3.

3.2.1.2   Effects of Kn on direct shoot proliferation

Shoot proliferation rate from nodes and internodes and was low compared to BAP when Kn was used singly in the culture media in different concentration. Highest percentage of proliferated shoots from the nodes was recorded 51.20±0.76 with Kn (2.0 mg /l) in the culture media. Average number of shoots per explant was 3.37 ± 0.48 and average length of the longest shoot was 2.64 ± 0.23 cm. But in the same concentration of Kn, highest percentage of proliferated shoots from the internodes was 40.25 ± 0.79, average number of shoot per explant was 3.21 ± 0.11 and average length of the longest shoot was 2.33 ± 0.39 cm. Other concentrations of Kn showed average results. The effects of different concentration of Kn on direct shoot proliferation from the different explants are presented by histogram graph in Fig. 4.

Table 5. Effects of different concentration of BAP and Kn on direct shoot proliferation from the different explants of *Stevia rebaudiana*.

Treatment (mg/l)		Percentage of explants showing proliferation		Average number of shoots per explant		Average length of the longest shoots (cm)	
		Nodes	Internodes	Nodes	Internodes	Nodes	Internodes
BAP	0.5	26.20 ± 1.90	23.55 ± 0.77	2.80 ± 0.28	2.48 ± 0.17	2.18 ± 0.11	2.10 ± 0.61
	1.0	38.60 ± 0.68	32.67 ± 0.24	3.17 ± 0.23	3.02 ± 0.34	2.27 ± 0.54	2.18 ± 0.28
	1.5	49.45 ± 0.35	41.53 ± 0.58	3.25 ± 0.39	3.19 ± 0.06	2.55 ± 0.32	2.30 ± 0.44
	2.0	**57.67 ± 0.57**	**44.49 ± 0.92**	**3.62 ± 0.12**	**3.47 ± 0.22**	**2.68 ± 0.21**	**2.32 ± 0.73**
	2.5	46.25 ± 0.80	40.12 ± 0.41	3.27 ± 0.53	3.22 ± 0.41	2.48 ± 0.38	2.27 ± 0.42
	3.0	34.40 ± 0.23	31.77 ± 0.47	3.10 ± 0.27	3.07 ± 0.57	2.30 ± 0.46	2.21 ± 0.75
Kn	0.5	22.55 ± 0.71	19.43 ± 0.12	2.65 ± 0.21	2.34 ± 0.08	2.19 ± 0.19	2.04 ± 0.38
	1.0	34.85 ± 1.23	30.77 ± 0.89	3.06 ± 0.35	2.79 ± 0.21	2.35 ± 0.30	2.22 ± 0.12
	1.5	45.24 ± 0.88	34.28 ± 0.63	3.10 ± 0.60	3.08 ± 0.43	2.52 ± 0.49	2.27 ± 0.35
	2.0	**51.20 ± 0.76**	**40.25 ± 0.79**	**3.37 ± 0.48**	**3.21 ± 0.11**	**2.64 ± 0.23**	**2.33 ± 0.39**
	2.5	42.65 ± 0.13	33.08 ± 0.35	3.05 ± 0.33	2.59 ± 0.27	2.51 ± 0.42	2.26 ± 0.66
	3.0	30.55 ± 0.45	28.11 ± 1.48	2.88 ± 0.29	2.48 ± 0.04	2.38 ± 0.15	2.19 ± 0.29
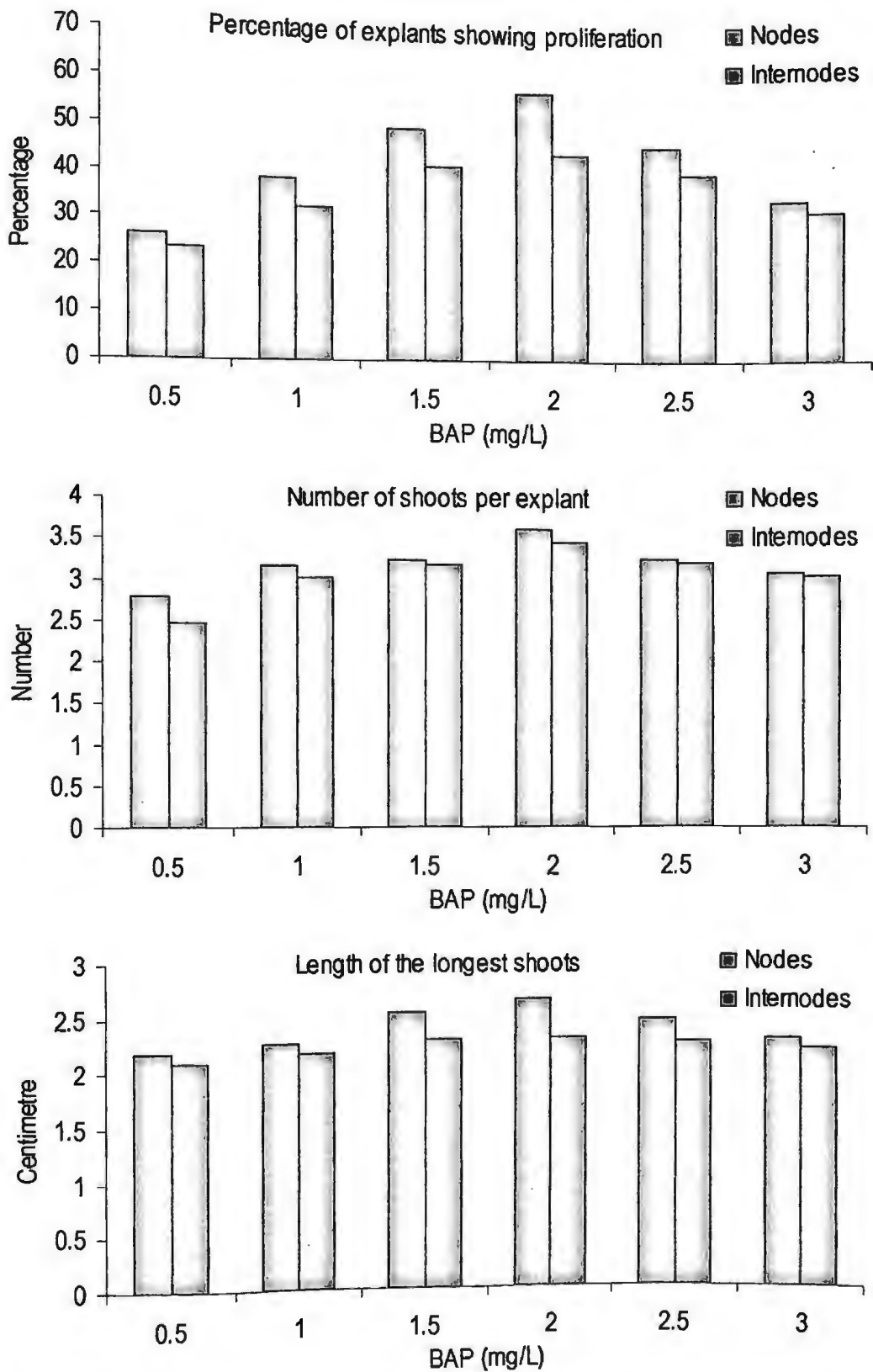
Fig. 3. Effects of different concentration of BAP on direct shoot proliferation from the different explants of *Stevia rebaudiana*; percentage proliferation (upper), number of shoots per explants (middle), length of longest shoot (lower).

Fig. 3. Effects of different concentration of BAP on direct shoot proliferation from the different explants of *Stevia rebaudiana*; percentage proliferation (upper), number of shoots per explants (middle), length of longest shoot (lower).

Fig. 4. Effects of different concentration of Kn on direct shoot proliferation from the different explants of *Stevia rebaudiana;* percentage proliferation (upper), number of shoots per explants (middle), length of longest shoot (lower).

3.2.2   Effects of combined growth regulators on direct shoot proliferation from the different explants

From the results of effects of individual growth regulators on direct shoot proliferation from the different explants (**3.2.1**) it is clear that Kn is less effective than BAP on direct shoot proliferation from nodes and internodes. To find out the effects of combined growth regulators on direct shoot proliferation from nodes and internodes, different concentration of Cytokinins (BAP) with different concentration of Auxins (IAA, IBA, NAA) were used in different combinations. Percentage of shoot proliferation from different explants was much better when Cytokinins and Auxins were used in combination in the culture media compared to their individual effects on direct shoot proliferation and combination of BAP and NAA in the culture media was found the most effective on direct shoot proliferation from the different explants.

3.2.2.1   Effects of different concentration of BAP with different concentration of IAA

Four different concentrations of BAP (1.0 mg/l, 1.5 mg/l, 2.0 mg/l, 2.5 mg/l) with five different concentrations of IAA (0.1 mg/l, 0.2 mg/l, 0.3 mg/l, 0.4 mg/l, 0.5 mg/l) were used in the culture media to find out the combined effects of BAP and IAA on direct shoots proliferation from nodes and internodes and the results are presented in Table 6. In most of the cases IAA (0.2 mg/l) or IAA (0.3 mg/l) with different concentration of BAP showed the best result.

BAP (1.0 mg/l) with five different concentration of IAA: In case of nodes as explants the highest percentage of proliferated shoots was observed when BAP (1.0 mg/l) + IAA (0.3 mg/l) was used in the culture media which was 69.26 ± 0.38. In this combination the average number of shoots per explant was 3.32 ± 0.08 and average length of the longest shoots was 2.46 ± 0.57 cm. But in case of internodes, the highest percentage of proliferated shoots was 58.33 ± 0.25 in same combination of the growth regulators and the average number of shoots per explant was 3.17 ± 0.27 and average length of the longest

shoots was 2.35 ± 0.43 cm. Other combinations showed average performances of shoot proliferation of the explants. The effects of BAP (1.0 mg/l) with different concentration of IAA on direct shoot proliferation from the different explants are presented by histogram in Fig. 5.

BAP (1.5 mg/l) with five different concentration of IAA: When BAP (1.5 mg/l) + IAA (0.2 mg/l) was used in the culture media, highest percentage of proliferated shoots was recorded from the nodes which was 72.48 ± 0.43, average number of shoots per explant was 3.49 ± 0.28 and average length of the longest shoots was 2.49 ± 0.11 cm. But when BAP (1.5 mg/l) + IAA (0.3 mg/l) was used, the highest percentage of proliferated shoots from the internodes was 66.17 ± 0.29 and average number of shoots per explant was 3.26 ± 0.16 and average length of the longest shoots was 2.41 ± 0.77 cm. Other combinations showed average performances of shoot proliferation of the explants. The effects of BAP (1.5 mg/l) with different concentration of IAA on direct shoot proliferation from the different explants are presented by histogram in Fig. 6.

BAP (2.0 mg/l) with five different concentration of IAA: BAP (2.0 mg/l) + IAA (0.2 mg/l) in the culture media showed the highest percentage of proliferated shoots from the nodes which was 76.48 ± 0.24.This was the best result among the BAP + IAA combinations. In this combination average number of shoots per explant was 3.86 ± 0.66 and average length of the longest shoots was 2.52 ± 0.41 cm. In the same combination of the growth regulators highest percentage of proliferated shoots from the internodes was 71.60 ± 1.03, average number of shoots per explant was 3.72±0.19 and average length of the longest shoots was 2.36 ± 0.53 cm. Other combinations showed average performances of shoot proliferation of the explants. The effects of BAP (2.0 mg/l) with different concentration of IAA on direct shoot proliferation from the different explants are presented by histogram in Fig. 7.

BAP (2.5 mg/l) with five different concentration of IAA: Highest percentage of proliferated shoots was found from the nodes which was 71.28 ± 0.15 when BAP (2.5 mg/l) + IAA (0.2 mg/l) was used in the culture media and average number of shoots per explant

was 3.41 ± 0.34 and average length of the longest shoots was 2.56 ± 0.49 cm. In same combination of the growth regulators highest percentage of proliferated shoots from the internodes was 66.24 ± 0.11, average number of shoots per explant was 3.27 ± 0.18 and average length of the longest shoots was 2.40 ± 0.25 cm. Other combinations showed average performances of shoot proliferation of the explants. The effects of BAP (2.5 mg/l) with different concentration of IAA on direct shoot proliferation from the different explants are presented by histogram in Fig. 8.

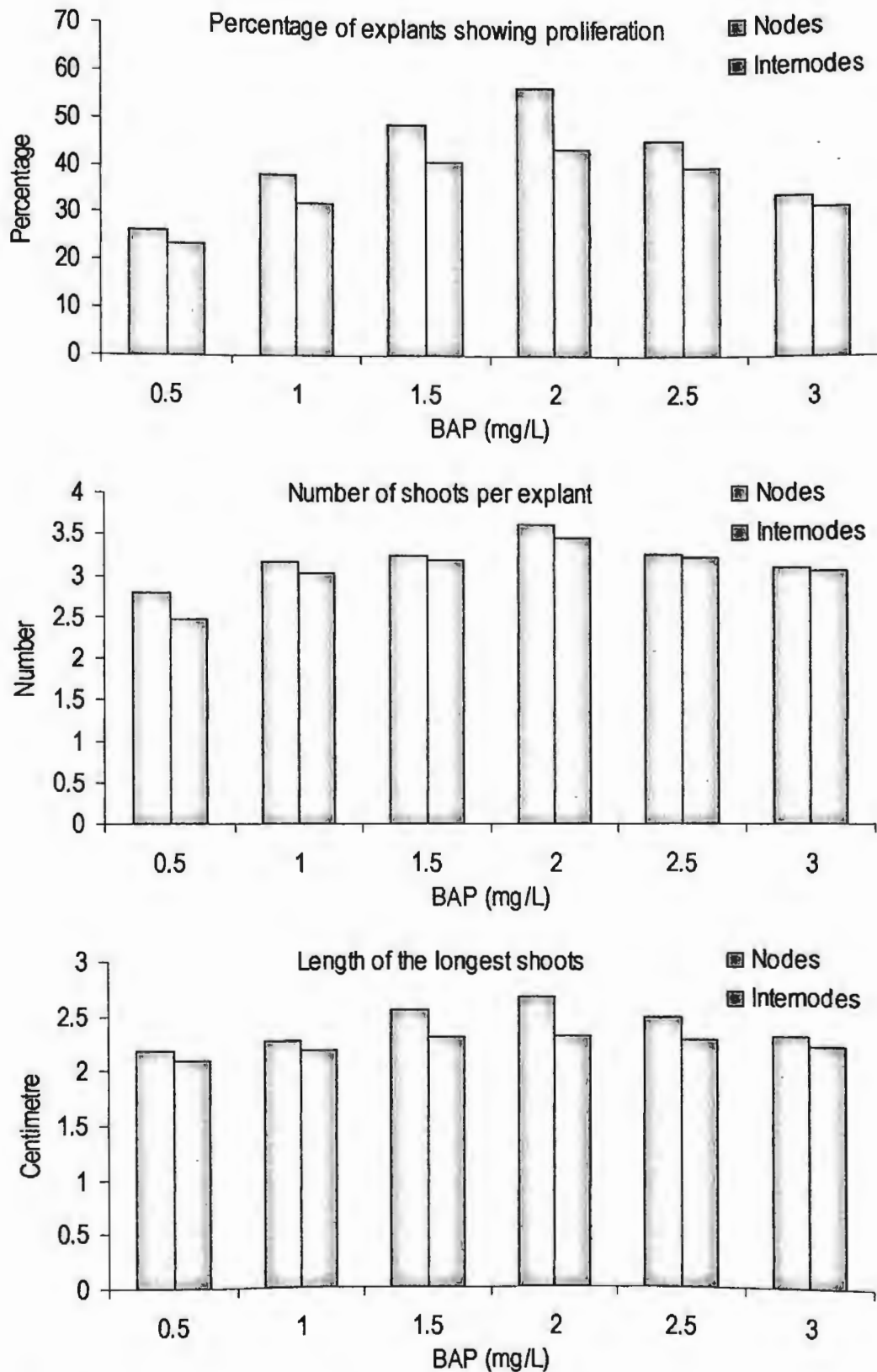**Table 6.** Effects of different concentration of BAP with different concentration of IAA on direct shoot proliferation from the different explants of *Stevia rebaudiana*.

Treatment (mg/l)		Percentage of explants showing proliferation		Average number of shoots per explant		Average length of the longest shoots (cm)	
BAP	IAA	Nodes	Internodes	Nodes	Internodes	Nodes	Internodes
1.0	0.1	61.45 ± 0.29	48.75 ± 0.87	3.29 ± 0.38	3.10 ± 0.91	2.31 ± 0.54	2.17 ± 0.80
	0.2	65.72 ± 0.54	54.16 ± 0.31	3.44 ± 0.51	3.28 ± 0.56	2.43 ± 0.29	2.32 ± 0.37
	0.3	69.26 ± 0.38	58.33 ± 0.25	3.32 ± 0.08	3.17 ± 0.27	2.46 ± 0.57	2.35 ± 0.43
	0.4	64.55 ± 1.08	52.48 ± 0.58	3.26 ± 0.23	3.05 ± 0.62	2.38 ± 0.88	2.20 ± 0.26
	0.5	59.24 ± 0.21	47.82 ± 1.10	3.19 ± 0.43	2.88 ± 0.34	2.34 ± 0.39	2.18 ± 0.14
1.5	0.1	67.60 ± 0.18	61.44 ± 0.23	3.34 ± 0.67	3.18 ± 0.77	2.35 ± 0.78	2.21 ± 0.71
	0.2	72.48 ± 0.43	64.86 ± 0.43	3.49 ± 0.28	3.36 ± 0.32	2.49 ± 0.11	2.36 ± 0.48
	0.3	71.25 ± 0.61	66.17 ± 0.29	3.38 ± 0.35	3.26 ± 0.16	2.53 ± 0.35	2.41 ± 0.77
	0.4	65.35 ± 0.26	60.42 ± 0.54	3.31 ± 0.51	3.14 ± 0.38	2.44 ± 0.73	2.31 ± 0.90
	0.5	62.84 ± 0.57	57.67 ± 0.35	3.26 ± 0.22	3.08 ± 0.45	2.37 ± 0.18	2.20 ± 0.34
2.0	0.1	70.15 ± 1.49	65.95 ± 0.18	3.70 ± 0.49	3.56 ± 0.13	2.41 ± 0.94	2.27 ± 0.12
	0.2	76.48 ± 0.24	71.60 ± 1.03	3.86 ± 0.66	3.72 ± 0.19	2.52 ± 0.41	2.36 ± 0.53
	0.3	73.61 ± 0.38	67.33 ± 0.33	3.76 ± 0.20	3.60 ± 0.25	2.63 ± 0.57	2.48 ± 0.76
	0.4	70.33 ± 0.74	65.46 ± 0.78	3.69 ± 0.53	3.52 ± 0.88	2.55 ± 0.44	2.41 ± 0.34
	0.5	67.84 ± 1.02	61.25 ± 0.19	3.64 ± 0.17	3.46 ± 0.34	2.47 ± 0.82	2.29 ± 0.23
2.5	0.1	64.72 ± 0.55	58.47 ± 0.48	3.34 ± 0.24	3.16 ± 0.82	2.36 ± 0.31	2.18 ± 0.66
	0.2	71.28 ± 0.15	66.24 ± 0.11	3.41 ± 0.34	3.27 ± 0.18	2.56 ± 0.49	2.40 ± 0.25
	0.3	67.55 ± 0.26	63.40 ± 0.47	3.32 ± 0.55	3.18 ± 0.26	2.48 ± 0.17	2.33 ± 0.31
	0.4	63.79 ± 0.93	59.67 ± 1.24	3.27 ± 0.79	3.10 ± 0.35	2.43 ± 0.57	2.24 ± 0.93
	0.5	62.47 ± 0.77	56.17 ± 0.85	3.23 ± 0.57	3.05 ± 0.16	2.34 ± 0.41	2.16 ± 0.64

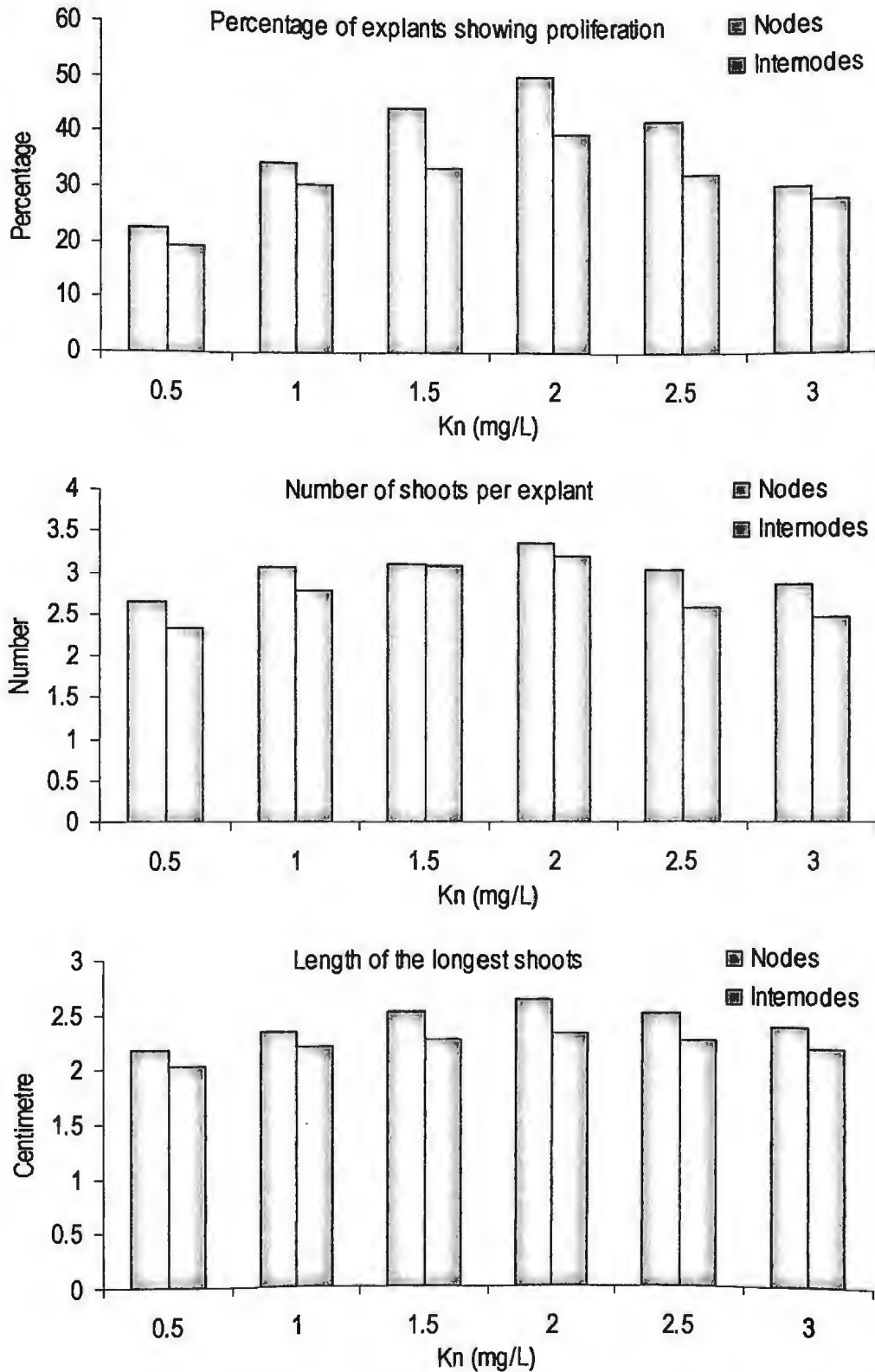**Fig. 5.** Effects of BAP (1.0 mg/l) with different concentration of IAA on direct shoot proliferation from the different explants of *Stevia rebaudiana*; percentage proliferation (upper), number of shoots per explants (middle), length of longest shoot (lower).
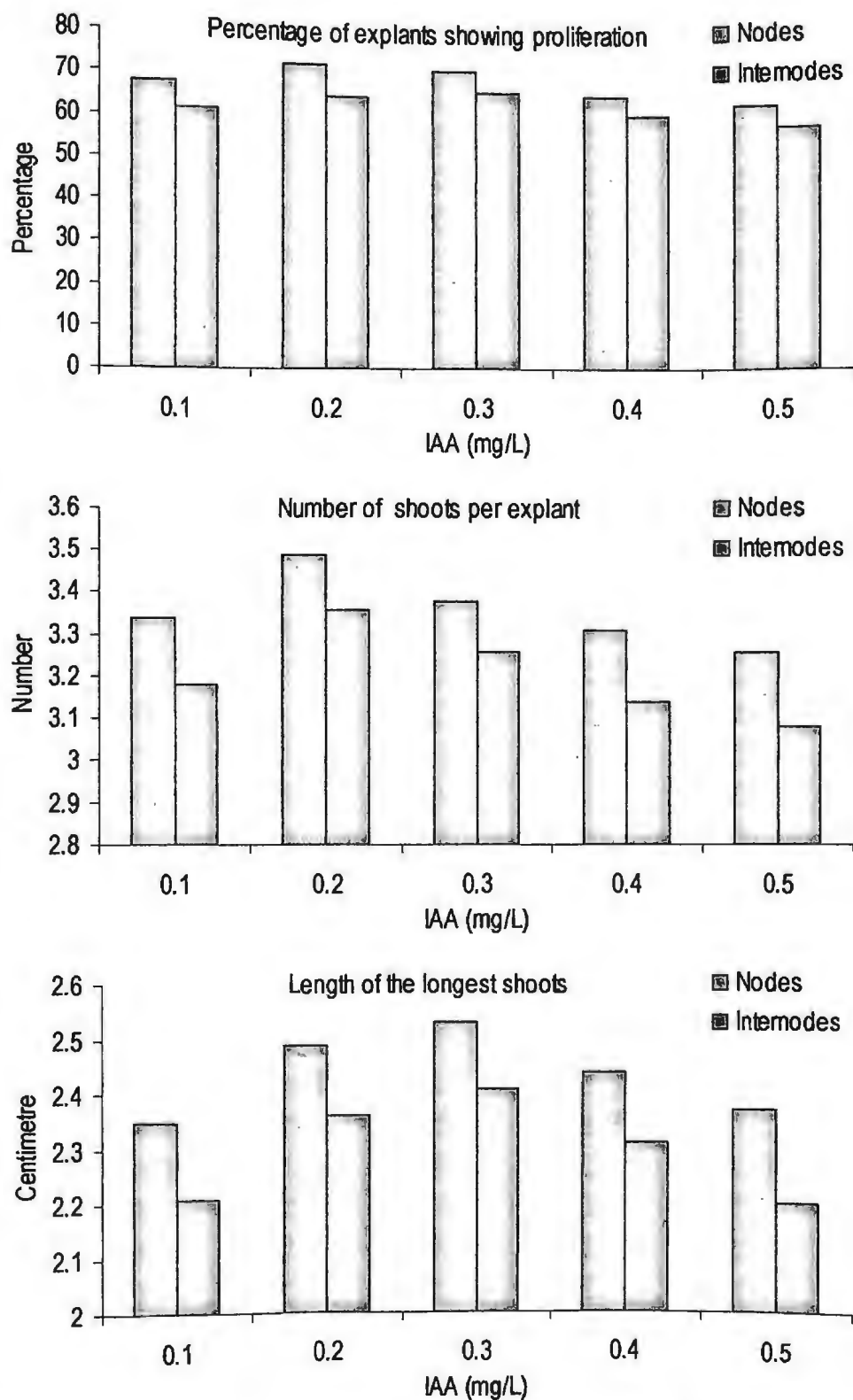
Fig. 6. Effects of BAP (1.5 mg/l) with different concentration of IAA on direct shoot proliferation from the different explants of *Stevia rebaudiana*; percentage proliferation (upper), number of shoots per explants (middle), length of longest shoot (lower).
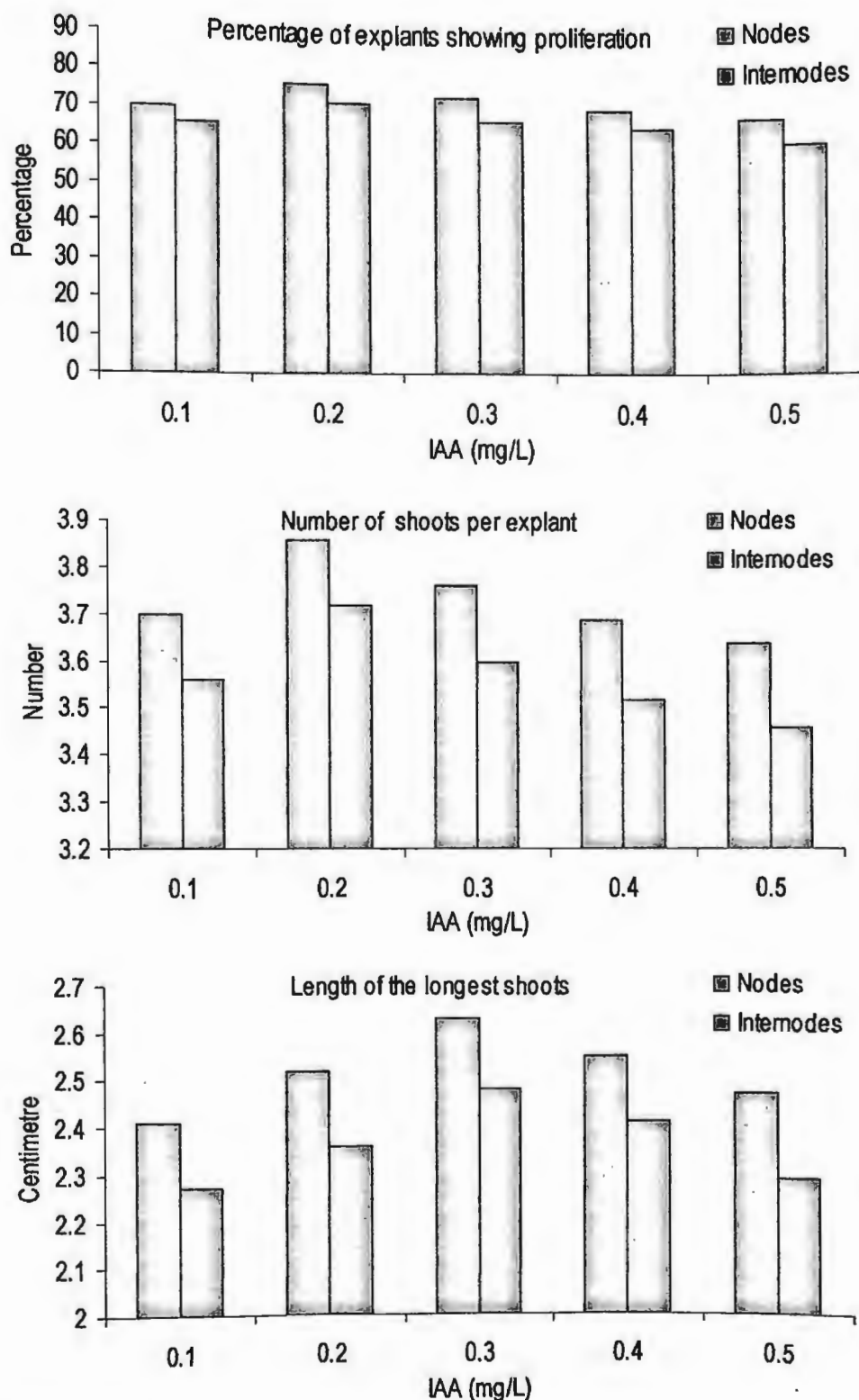
Fig. 7. Effects of BAP (2.0 mg/l) with different concentration of IAA on direct shoot proliferation from the different explants of *Stevia rebaudiana*; percentage proliferation (upper), number of shoots per explants (middle), length of longest shoot (lower).

Fig. 8. Effects of BAP (2.5 mg/l) with different concentration of IAA on direct shoot proliferation from the different explants of *Stevia rebaudiana*; percentage proliferation (upper), number of shoots per explants (middle), length of longest shoot (lower).

3.2.2.2 Effects of different concentration of BAP with different concentration of IBA

Four different concentrations of BAP (1.0 mg/l, 1.5 mg/l, 2.0 mg/l, 2.5 mg/l) with five different concentrations of IBA (0.1 mg/l, 0.2 mg/l, 0.3 mg/l, 0.4 mg/l, 0.5 mg/l) were used in the culture media to find out the combined effects of BAP and IBA on direct shoots proliferation from nodes and internodes and the results are presented in Table 7. In most of the cases IBA (0.3 mg/l) with different concentration of BAP showed the best result.

BAP (1.0 mg/l) with five different concentration of IBA: In case of nodes as explants the highest percentage of proliferated shoots was observed when BAP (1.0 mg/l) + IBA (0.3 mg/l) was used in the culture media which was 70.37 ± 0.35. In this combination average number of shoots per explant was 3.38 ± 0.26 and average length of the longest shoots was 2.43 ± 0.38 cm. But in case of internodes, the highest percentage of proliferated shoots was 59.68 ± 0.89 in same combination of the growth regulators and average number of shoots per explant was 3.15 ± 0.19 and average length of the longest shoots was 2.43 ± 0.41 cm. Other combinations showed average performances of shoot proliferation of the explants. The effects of BAP (1.0 mg/l) with different concentration of IBA on direct shoot proliferation from the different explants are presented by histogram in Fig. 9.

BAP (1.5 mg/l) with five different concentration of IBA: When BAP (1.5 mg/l) + IBA (0.2 mg/l) was used in the culture media, highest percentage of proliferated shoots was recorded from the nodes which was 73.15 ± 0.43, average number of shoots per explant was 3.51 ± 0.15 and average length of the longest shoots was 2.48 ± 0.43 cm. The highest percentage of proliferated shoots from the internodes was 63.42 ± 0.22 in same combination of the growth regulators and average number of shoots per explant was 3.33 ± 0.58 and average length of the longest shoots was 2.48 ± 0.10 cm. Other combinations showed average performances of shoot proliferation of the explants. The effects of BAP (1.5 mg/l) with different concentration of IBA on direct shoot proliferation from the different explants are presented by histogram in Fig. 10.
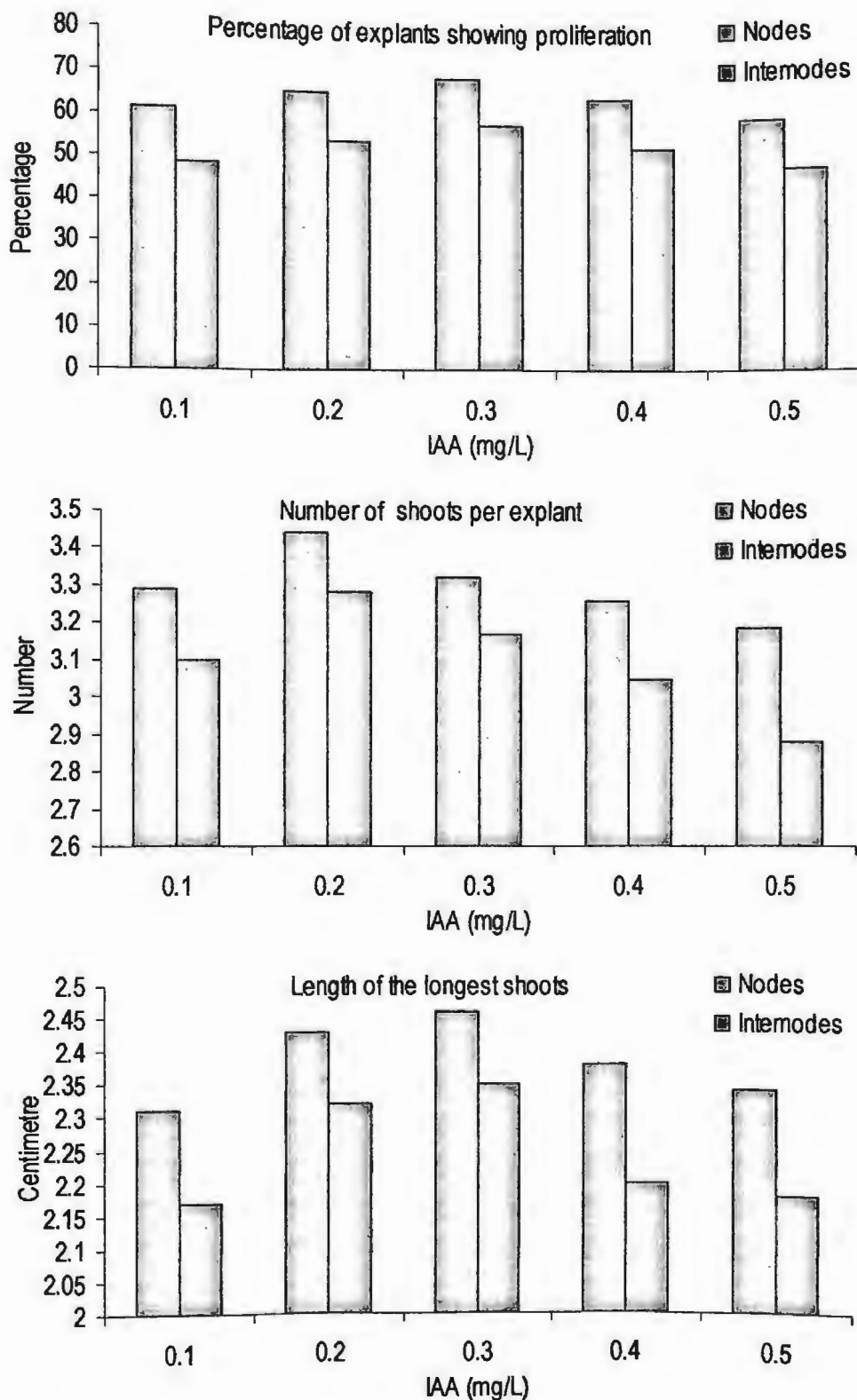
BAP (2.0 mg/l) with five different concentration of IBA: BAP (2.0 mg/l) + IBA (0.2 mg/l) in the culture media showed the highest percentage of proliferated shoots from the nodes which was 79.52 ± 0.79.This was the best result among the BAP + IBA combinations. In this combination average number of shoots per explant was 3.92 ± 0.56 and average length of the longest shoots was 2.54 ± 0.49 cm. In the same combination of the growth regulators highest percentage of proliferated shoots from the internodes was 71.68 ± 0.18, average number of shoots per explant was 3.60 ± 0.31 and average length of the longest shoots was 2.54 ± 0.57 cm. Other combinations showed average performances of shoot proliferation of the explants. The effects of BAP (2.0 mg/l) with different concentration of IBA on direct shoot proliferation from the different explants are presented by histogram in Fig. 11.

BAP (2.5 mg/l) with five different concentration of IBA: Highest percentage of proliferated shoots was found from the nodes which was 73.84 ± 1.31 when BAP (2.5 mg/l) + IBA (0.2 mg/l) was used in the culture media and average number of shoots per explant was 3.43 ± 0.82 and average length of the longest shoots was 2.53 ± 0.16 cm. In same combination of the growth regulators highest percentage of proliferated shoots from the internodes was 64.75 ± 0.55, average number of shoots per explant was 3.35 ± 0.43 and average length of the longest shoots was 2.53 ± 0.27 cm. Other combinations showed average performances of shoot proliferation of the explants. The effects of BAP (2.5 mg/l) with different concentration of IBA on direct shoot proliferation from the different explants are presented by histogram in Fig. 12.

Table 7. Effects of different concentration of BAP with different concentration of IBA on direct shoot proliferation from the different explants of *Stevia rebaudiana*.

| Treatment (mg/L)		Percentage of explants showing proliferation		Average number of shoots per explant		Average length of the longest shoots (cm)	
BAP	IBA	Nodes	Internodes	Nodes	Internodes	Nodes	Internodes
1.0	0.1	62.54 ± 1.32	50.28 ± 0.44	3.34 ± 0.12	2.95 ± 0.47	2.28 ± 0.57	2.28 ± 0.70
	0.2	65.84 ± 0.87	55.35 ± 0.68	3.47 ± 0.39	3.20 ± 0.13	2.42 ± 0.92	2.42 ± 0.26
	0.3	70.37 ± 0.35	59.68 ± 0.89	3.38 ± 0.26	3.15 ± 0.19	2.43 ± 0.38	2.43 ± 0.41
	0.4	63.17 ± 0.22	51.20 ± 1.06	3.31 ± 0.31	3.08 ± 0.44	2.35 ± 0.19	2.35 ± 0.87
	0.5	60.28 ± 0.97	48.62 ± 0.47	3.24 ± 0.28	2.87 ± 0.38	2.31 ± 0.31	2.31 ± 0.23
1.5	0.1	66.47 ± 0.85	58.71 ± 0.92	3.38 ± 0.19	3.15 ± 0.49	2.32 ± 0.08	2.32 ± 0.44
	0.2	73.15 ± 0.43	63.42 ± 0.22	3.51 ± 0.15	3.33 ± 0.58	2.48 ± 0.43	2.48 ± 0.10
	0.3	70.67 ± 0.28	61.33 ± 0.48	3.41 ± 0.33	3.26 ± 0.36	2.50 ± 0.68	2.50 ± 0.38
	0.4	65.92 ± 0.76	54.83 ± 0.53	3.29 ± 0.51	3.16 ± 0.22	2.39 ± 0.71	2.39 ± 0.57
	0.5	63.25 ± 1.24	52.46 ± 0.27	3.27 ± 0.36	3.10 ± 0.39	2.31 ± 0.63	2.31 ± 0.62
2.0	0.1	72.80 ± 0.65	61.17 ± 0.81	3.67 ± 0.24	3.42 ± 0.44	2.37 ± 0.25	2.37 ± 0.38
	0.2	79.52 ± 0.79	71.68 ± 0.18	3.92 ± 0.56	3.60 ± 0.31	2.54 ± 0.49	2.54 ± 0.57
	0.3	74.39 ± 0.33	64.38 ± 1.70	3.79 ± 0.18	3.48 ± 0.15	2.57 ± 0.33	2.57 ± 0.23
	0.4	71.63 ± 0.21	60.42 ± 0.24	3.74 ± 0.25	3.36 ± 0.34	2.49 ± 0.29	2.49 ± 0.36
	0.5	70.82 ± 0.55	59.67 ± 0.98	3.69 ± 0.31	3.30 ± 0.79	2.40 ± 0.54	2.40 ± 0.53
2.5	0.1	67.25 ± 0.28	56.42 ± 1.39	3.31 ± 0.64	3.20 ± 0.16	2.36 ± 0.30	2.36 ± 0.48
	0.2	73.84 ± 1.31	64.75 ± 0.55	3.43 ± 0.82	3.35 ± 0.43	2.53 ± 0.16	2.53 ± 0.27
	0.3	70.42 ± 0.95	59.33 ± 0.68	3.30 ± 0.49	3.22 ± 0.23	2.51 ± 0.97	2.51 ± 0.58
	0.4	67.62 ± 0.36	57.40 ± 0.43	3.23 ± 0.28	3.15 ± 0.17	2.45 ± 0.28	2.45 ± 0.14
	0.5	64.89 ± 0.74	52.85 ± 0.66	3.18 ± 0.30	3.05 ± 0.42	2.29 ± 0.22	2.29 ± 0.39

Fig. 9. Effects of BAP (1.0 mg/l) with different concentration of IBA on direct shoot proliferation from the different explants of *Stevia rebaudiana*; percentage proliferation (upper), number of shoots per explants (middle), length of longest shoot (lower).
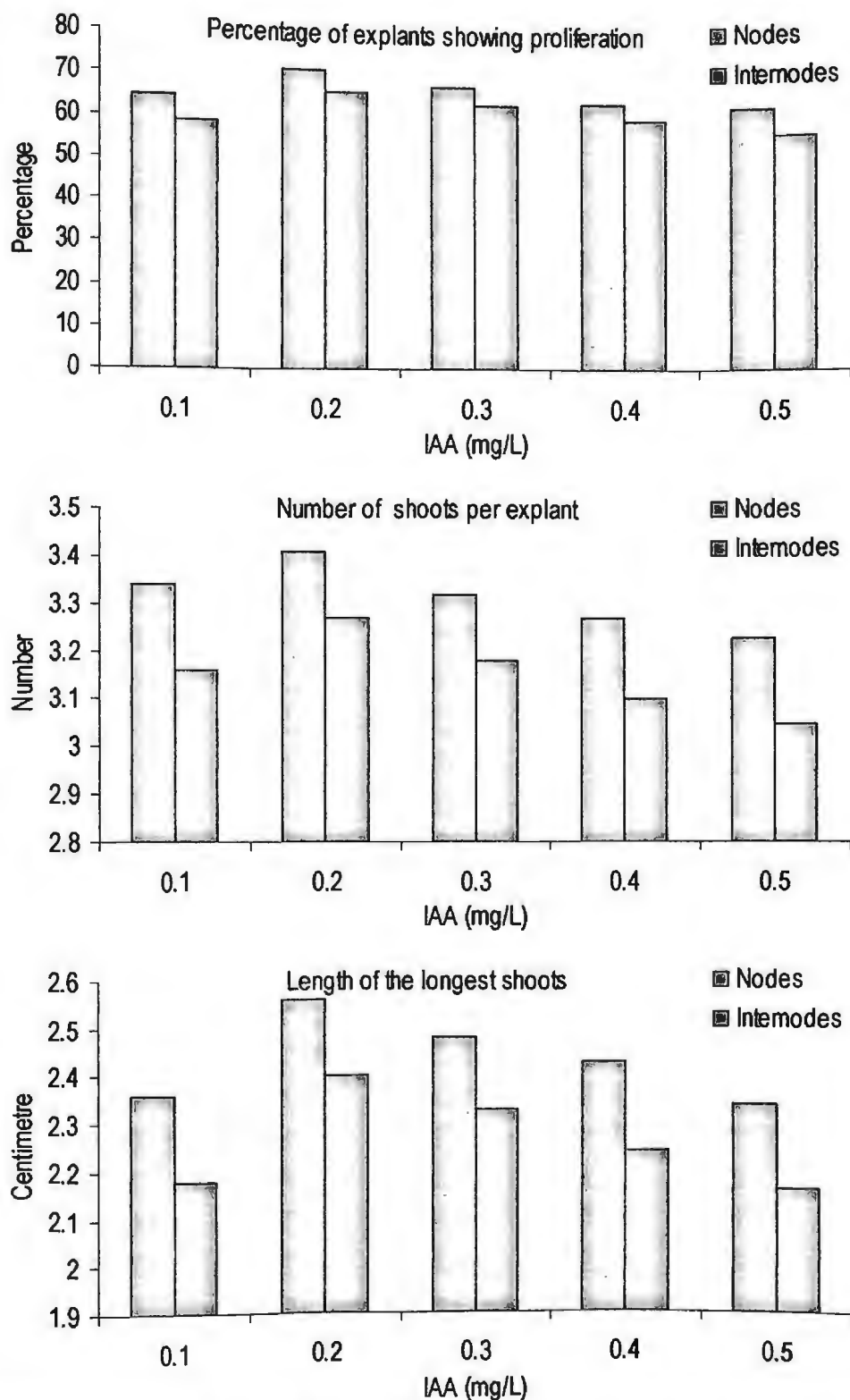
Fig. 10. Effects of BAP (1.5 mg/l) with different concentration of IBA on direct shoot proliferation from the different explants of *Stevia rebaudiana*; percentage proliferation (upper), number of shoots per explants (middle), length of longest shoot (lower).
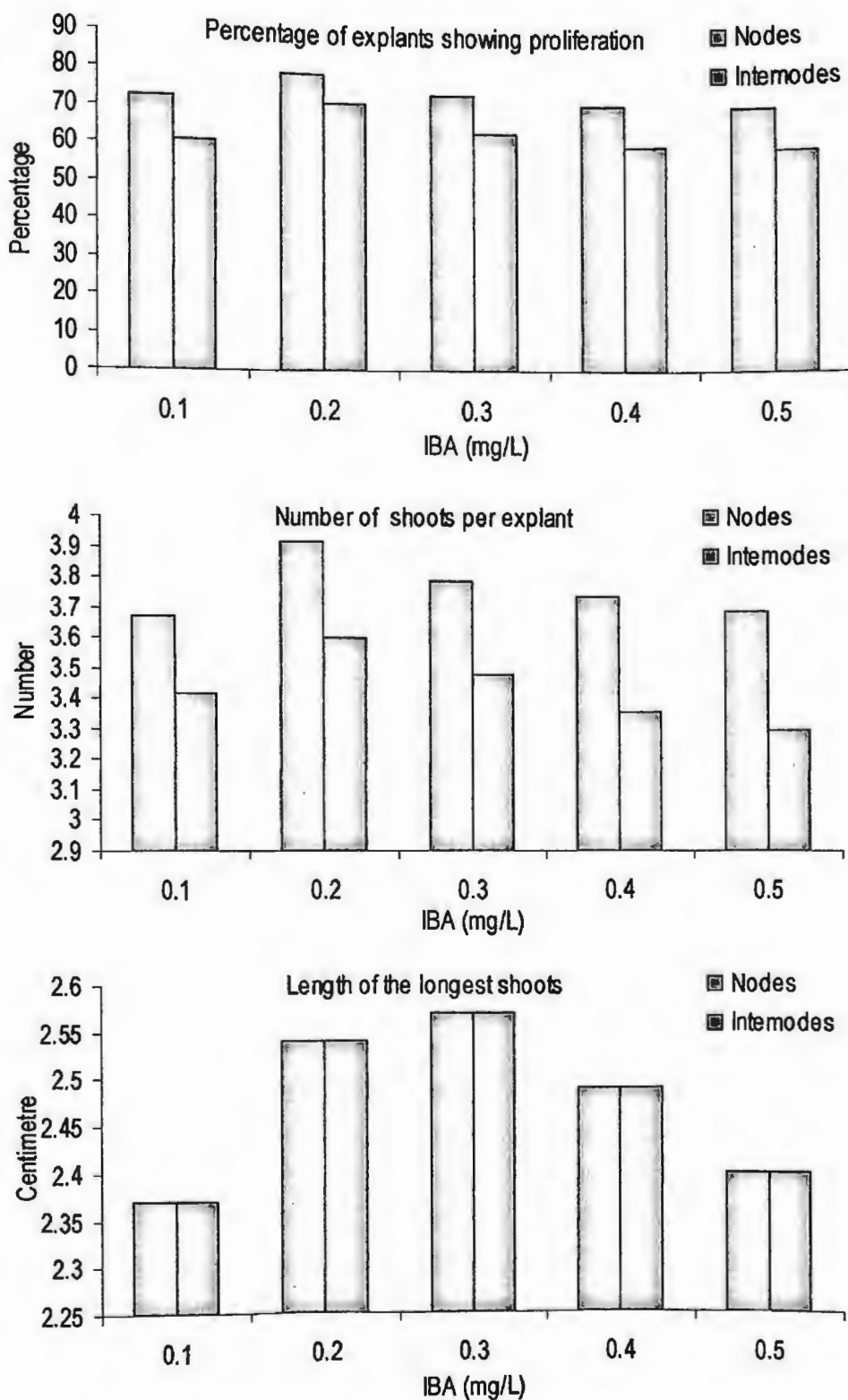
Fig. 11. Effects of BAP (2.0 mg/l) with different concentration of IBA on direct shoot proliferation from the different explants of *Stevia rebaudiana*; percentage proliferation (upper), number of shoots per explants (middle), length of longest shoot (lower).

Fig. 12. Effects of BAP (2.5 mg/l) with different concentration of IBA on direct shoot proliferation from the different explants of *Stevia rebaudiana*; percentage proliferation (upper), number of shoots per explants (middle), length of longest shoot (lower).

3.2.2.3 Effects of different concentration of BAP with different concentration of NAA

Four different concentrations of BAP (1.0 mg/l, 1.5 mg/l, 2.0 mg/l, 2.5 mg/l) with five different concentrations of NAA (0.1 mg/l, 0.2 mg/l, 0.3 mg/l, 0.4 mg/l, 0.5 mg/l) were used in the culture media to find out the combined effects of BAP and NAA on direct shoots proliferation from nodes and internodes and the results are presented in Table 8. In most of the cases NAA (0.2 mg/l) with different concentration of BAP showed the best result.

BAP (1.0 mg/l) with five different concentration of NAA: In case of nodes as explants the highest percentage of proliferated shoots was observed when BAP (1.0 mg/l) + NAA (0.2 mg/l) was used in the culture media which was 77.38 ± 0.96. In this combination average number of shoots per explant was 3.56 ± 0.45 and average length of the longest shoots was 2.55 ± 0.15 cm. But in case of internodes, the highest percentage of proliferated shoots was 67.75 ± 0.28 in same combination of the growth regulators and average number of shoots per explant was 3.33 ± 0.36 and average length of the longest shoots was 2.63 ± 0.70 cm. Other combinations showed average performances of shoot proliferation of the explants. The effects of BAP (1.0 mg/l) with different concentration of NAA on direct shoot proliferation from the different explants are presented by histogram in Fig. 13.
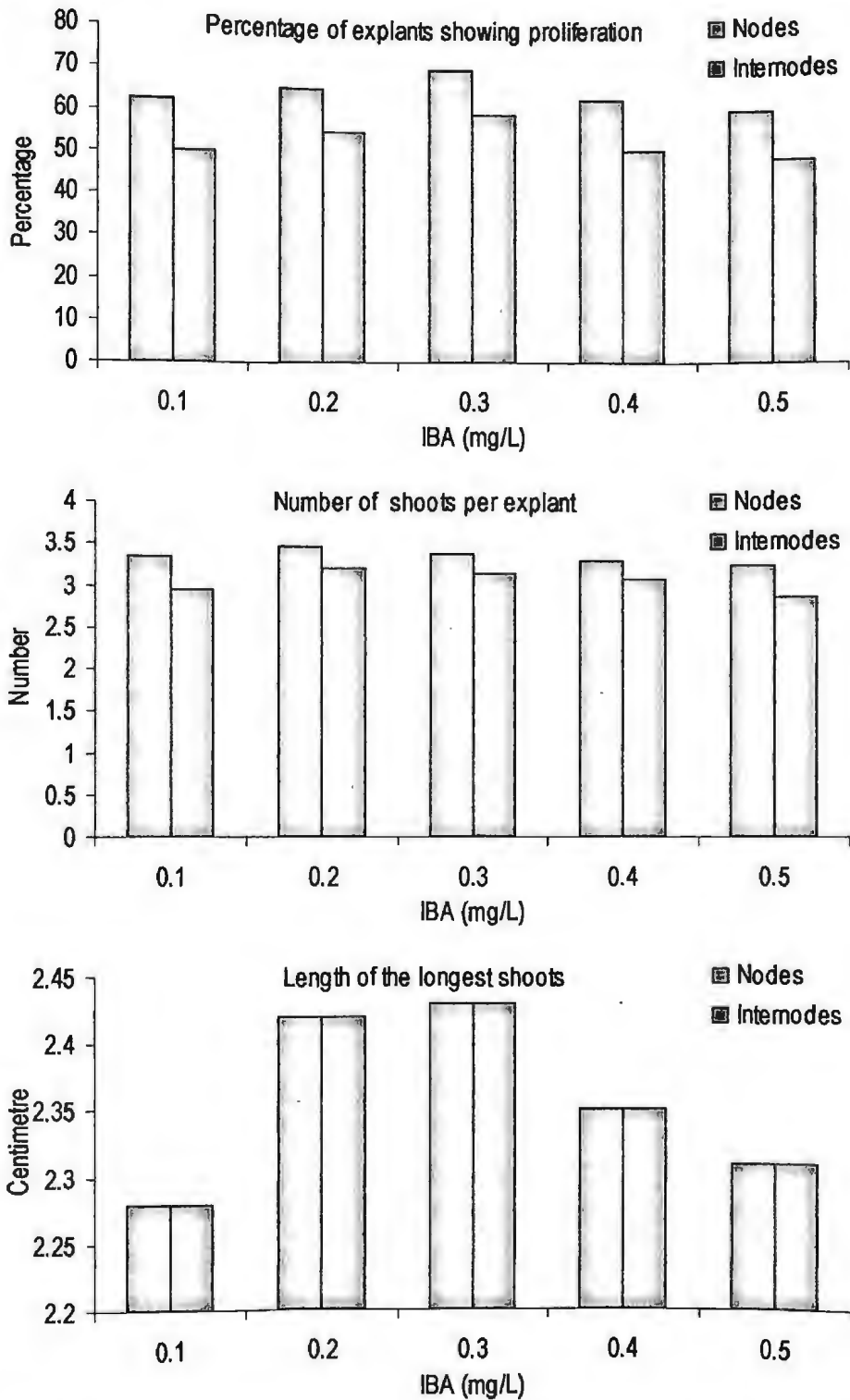
BAP (1.5 mg/l) with five different concentration of NAA: When BAP (1.5 mg/l) + NAA (0.2 mg/l) was used in the culture media, highest percentage of proliferated shoots was recorded from the nodes which was 81.44 ± 0.37, average number of shoots per explant was 3.64 ± 0.46 and average length of the longest shoots was 2.61 ± 0.44 cm. In case of internodes, the highest percentage of proliferated shoots was 73.33 ± 0.59 in same combination of the growth regulators and average number of shoots per explant was 3.45 ± 0.18 and average length of the longest shoots was 2.72 ± 0.31 cm. Other combinations showed average performances of shoot proliferation of the explants. The effects of BAP (1.5 mg/l) with different concentration of NAA on direct shoot proliferation from the different explants are presented by histogram in Fig. 14.
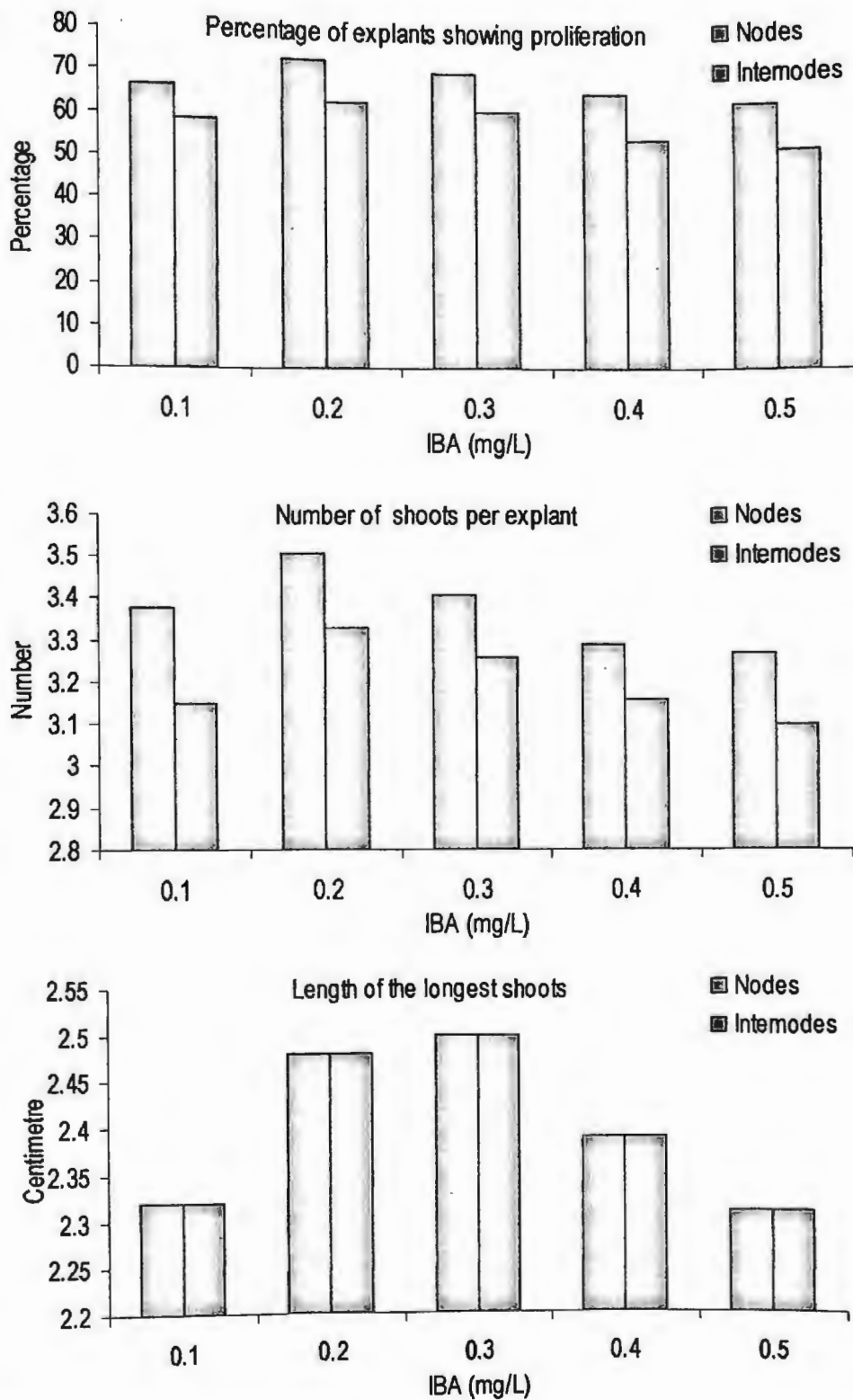
BAP (2.0 mg/l) with five different concentration of NAA: BAP (2.0 mg/l) + NAA (0.2 mg/l) in the culture media showed the highest percentage of proliferated shoots from the nodes which was 92.84 ± 0.45. This was the best result among the BAP + NAA combinations. In this combination average number of shoots per explant was 4.16 ± 0.28 and average length of the longest shoots was 2.67 ± 0.98 cm. In the same combination of the growth regulators highest percentage of proliferated shoots from the internodes was 84.87 ± 0.62, average number of shoots per explant was 3.95 ± 0.34 and average length of the longest shoots was 2.78 ± 0.43 cm. Other combinations showed average performances of shoot proliferation of the explants. The effects of BAP (2.0 mg/l) with different concentration of NAA on direct shoot proliferation from the different explants are presented by histogram in Fig. 15.

BAP (2.5 mg/l) with five different concentration of NAA: Highest percentage of proliferated shoots was found from the nodes which was 82.68 ± 0.57 when BAP (2.5 mg/l) + NAA (0.2 mg/l) was used in the culture media and average number of shoots per explant was 3.53 ± 0.43 and average length of the longest shoots was 2.57 ± 0.37 cm. In same combination of the growth regulators highest percentage of proliferated shoots from the internodes was 74.25 ± 0.29, average number of shoots per explant was 3.38 ± 0.16 and average length of the longest shoots was 2.63 ± 0.71 cm. Other combinations showed average performances of shoot proliferation of the explants. The effects of BAP (2.5 mg/l) with different concentration of NAA on direct shoot proliferation from the different explants are presented by histogram in Fig. 16.

Table 8. Effects of different concentration of BAP with different concentration of NAA on direct shoot proliferation from the different explants of *Stevia rebaudiana.*

Treatment (mg/l)		Percentage of explants showing proliferation		Average number of shoots per explant		Average length of the longest shoots (cm)	
BAP	NAA	Nodes	Internodes	Nodes	Internodes	Nodes	Internodes
	0.1	68.42 ± 0.48	56.17 ± 0.33	3.42 ± 0.61	3.20 ± 0.95	2.39 ± 0.58	2.46 ± 0.39
	0.2	77.38 ± 0.96	67.75 ± 0.28	3.56 ± 0.45	3.33 ± 0.36	2.55 ± 0.15	2.63 ± 0.70
1.0	0.3	72.65 ± 0.17	63.48 ± 0.56	3.44 ± 0.34	3.28 ± 0.28	2.47 ± 0.31	2.52 ± 0.23
	0.4	67.58 ± 1.34	58.20 ± 0.95	3.39 ± 0.29	3.20 ± 0.66	2.41 ± 0.48	2.45 ± 0.49
	0.5	64.15 ± 1.42	55.80 ± 0.87	3.31 ± 0.40	3.12 ± 0.37	2.36 ± 0.73	2.42 ± 0.55
	0.1	71.67 ± 0.22	61.78 ± 1.24	3.47 ± 0.29	3.28 ± 0.75	2.43 ± 0.40	2.49 ± 0.72
	0.2	81.44 ± 0.37	73.33 ± 0.59	3.64 ± 0.46	3.45 ± 0.18	2.61 ± 0.44	2.72 ± 0.31
1.5	0.3	78.20 ± 0.42	69.15 ± 0.39	3.52 ± 0.93	3.33 ± 0.06	2.52 ± 0.29	2.58 ± 0.38
	0.4	72.57 ± 1.33	63.45 ± 0.28	3.45 ± 0.22	3.25 ± 0.46	2.45 ± 0.76	2.52 ± 0.94
	0.5	67.26 ± 0.56	56.70 ± 1.47	3.37 ± 0.41	3.17 ± 0.78	2.39 ± 0.58	2.44 ± 0.53
	0.1	79.33 ± 0.80	67.48 ± 0.55	3.71 ± 0.57	3.52 ± 0.24	2.53 ± 0.14	2.58 ± 0.11
	0.2	92.84 ± 0.45	84.87 ± 0.62	4.16 ± 0.28	3.95 ± 0.34	2.67 ± 0.98	2.78 ± 0.43
2.0	0.3	86.39 ± 0.91	78.33 ± 0.48	3.96 ± 0.11	3.70 ± 0.76	2.60 ± 0.72	2.64 ± 0.81
	0.4	78.62 ± 1.22	70.45 ± 0.71	3.84 ± 0.49	3.65 ± 0.25	2.54 ± 0.05	2.58 ± 0.27
	0.5	74.45 ± 0.38	65.25 ± 0.53	3.70 ± 0.74	3.48 ± 0.87	2.49 ± 0.31	2.53 ± 0.48
	0.1	74.75 ± 0.94	62.90 ± 0.96	3.38 ± 0.27	3.22 ± 0.94	2.43 ± 0.69	2.49 ± 0.23
	0.2	82.68 ± 0.57	74.25 ± 0.29	3.53 ± 0.43	3.38 ± 0.16	2.57 ± 0.37	2.63 ± 0.71
2.5	0.3	77.45 ± 0.76	68.30 ± 0.51	3.41 ± 0.18	3.27 ± 0.77	2.49 ± 0.41	2.55 ± 0.30
	0.4	73.24 ± 0.25	64.76 ± 0.82	3.37 ± 0.74	3.20 ± 0.43	2.42 ± 0.28	2.48 ± 0.56
	0.5	67.89 ± 0.44	59.17 ± 0.48	3.29 ± 0.46	3.10 ± 0.25	2.36 ± 0.22	2.41 ± 0.38
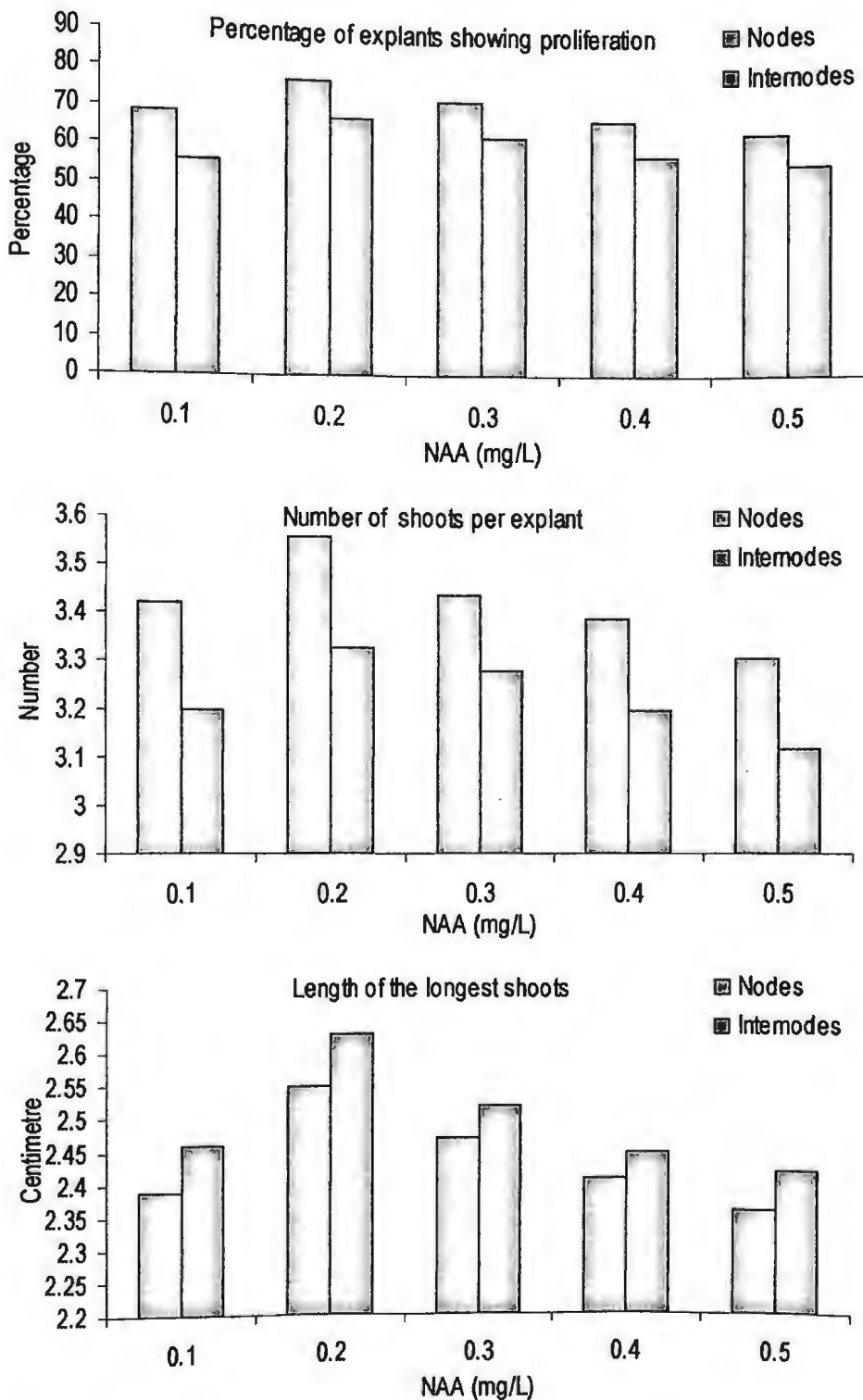
Fig. 13. Effects of BAP (1.0 mg/l) with different concentration of NAA on direct shoot proliferation from the different explants of *Stevia rebaudiana*; percentage proliferation (upper), number of shoots per explants (middle), length of longest shoot (lower).
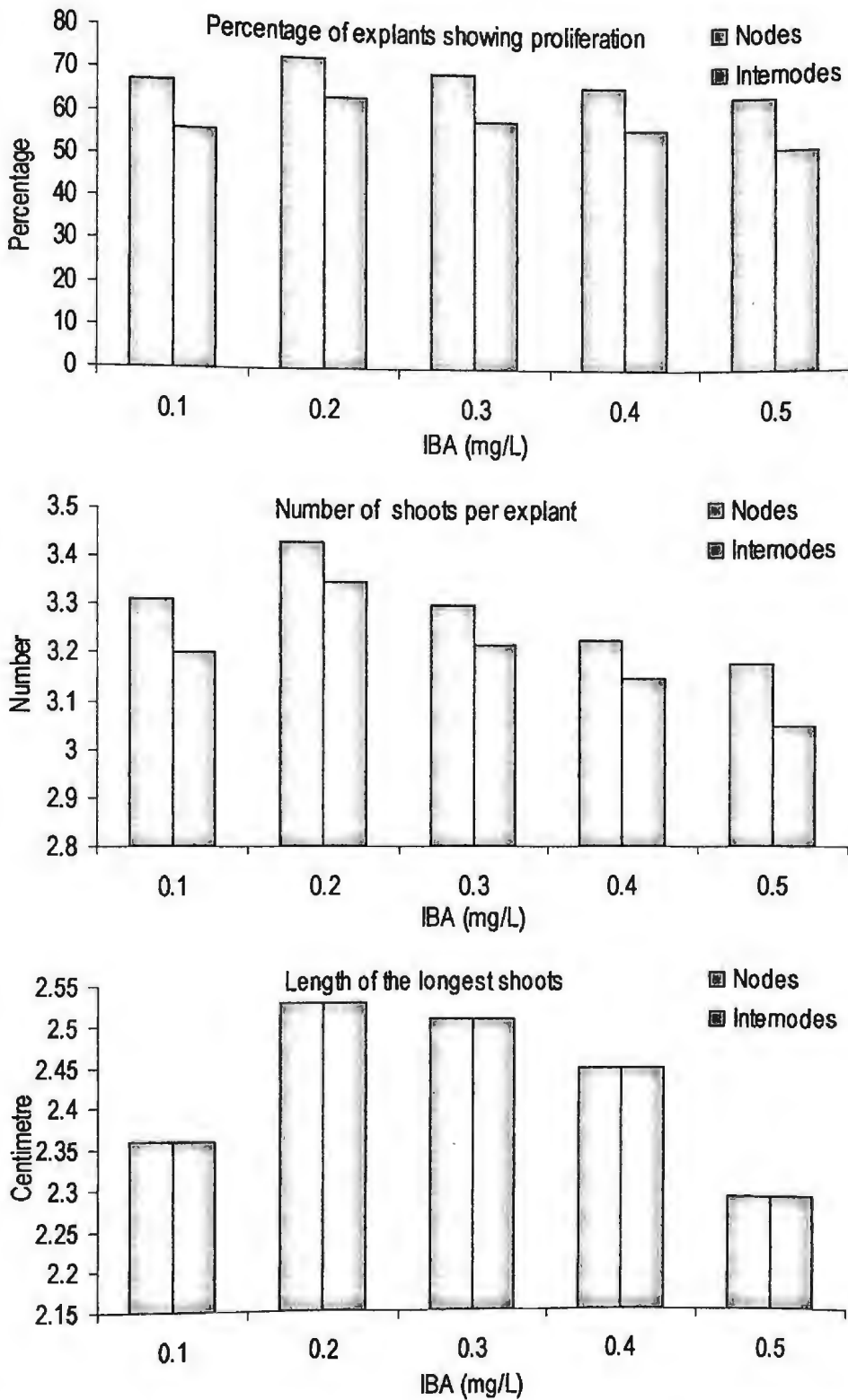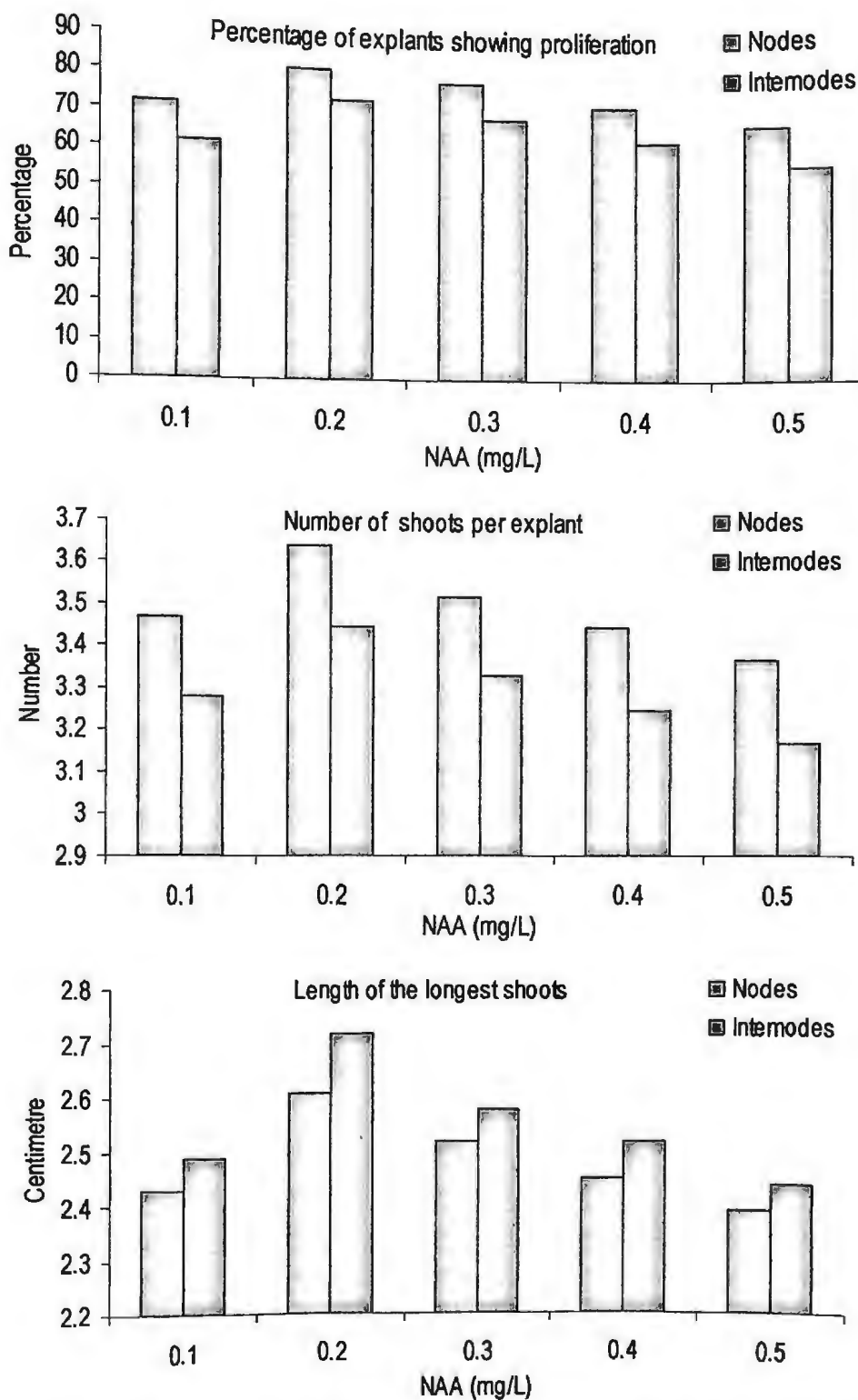
Fig. 14. Effects of BAP (1.5 mg/l) with different concentration of NAA on direct shoot proliferation from the different explants of *Stevia rebaudiana*; percentage proliferation (upper), number of shoots per explants (middle), length of longest shoot (lower).
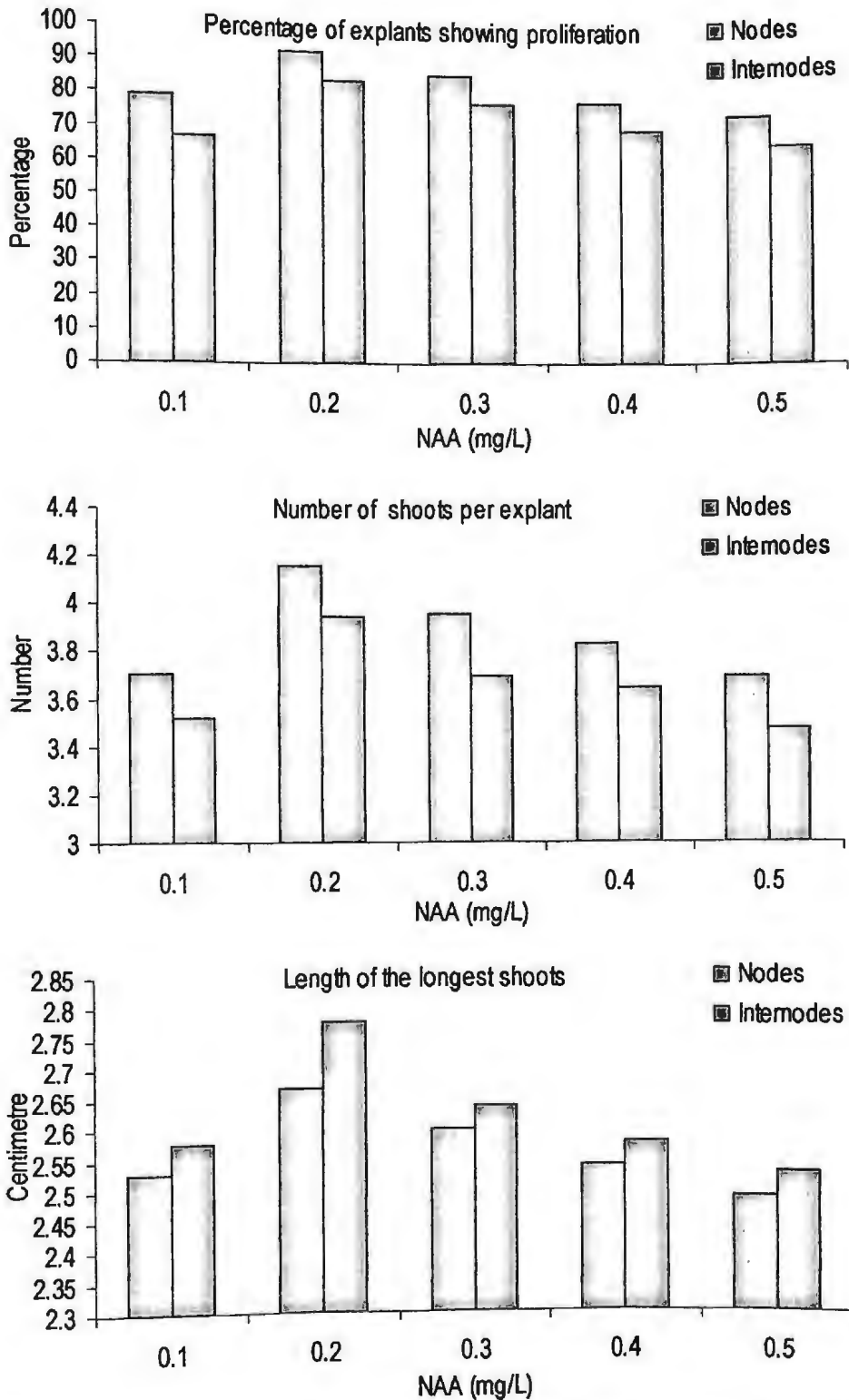
Fig. 15. Effects of BAP (2.0 mg/l) with different concentration of NAA on direct shoot proliferation from the different explants of *Stevia rebaudiana*; percentage proliferation (upper), number of shoots per explants (middle), length of longest shoot (lower).
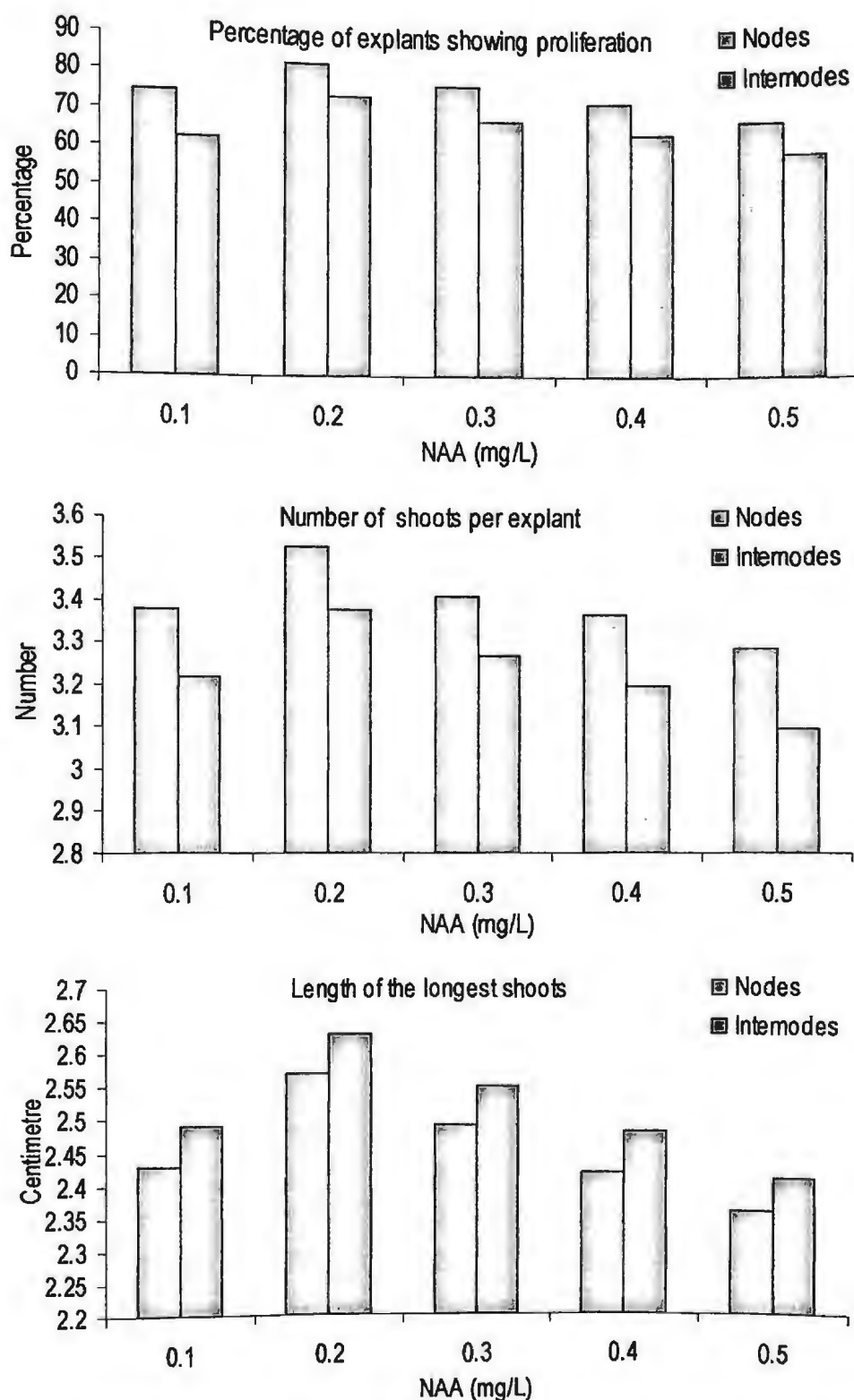
Fig. 16. Effects of BAP (2.5 mg/l) with different concentration of NAA on direct shoot proliferation from the different explants of *Stevia rebaudiana*; percentage proliferation (upper), number of shoots per explants (middle), length of longest shoot (lower).

Plate 2. Direct shoot proliferation from explants of *Stevia rebaudiana*

3.3   Callus formation from the different explants

Different concentration of Auxins (IAA, IBA, NAA and 2,4-D) were used individually and in combination in the culture media to observe the effects of the growth regulators on callus formation from nodes, internodes and leaves. In all cases internodes showed the highest percentage of callus formation compared to nodes and leaves (Plate 3).

3.3.1   Effects of individual growth regulators on callus formation from the different explants

Four different types of Auxins (IAA, IBA, NAA and 2,4-D), each with five different concentrations (1.0 mg/l, 2.0 mg/l, 3.0 mg/l, 4.0 mg/l, 5.0 mg/l) were used in the culture media to find out the effects of individual growth regulators on callus formation from nodes, internodes and leaves and the results are presented in Table 9. But all types of Auxins were found less effective on callus formation from nodes, internodes and leaves when they were used individually in the culture media.

3.3.1.1   Effects of IAA on callus formation from the different explants

Callus formation rate from nodes, internodes and leaves was very low when IAA was used singly in the culture media in different concentration. Only 18.54 ± 0.75 nodes showed callus formation in the culture media with IAA (4.0 mg/l). Average day of callus initiation was 13.85 ± 0.83 and colour of callus was brownish green. In case of internodes, percentage of callus formation was 22.70 ± 0.58, average day of callus initiation was 13.50 ± 0.49 and colour of callus was yellowish green. IAA (4.0 mg/l) in the culture media showed only 16.54 ± 1.08 leaves to form callus. Average day of callus initiation was 14.20 ± 0.18 and colour of callus was brownish green. The effects of different concentration of IAA on callus formation from the different explants are presented by histogram in Fig. 17.

3.3.1.2   Effects of IBA on callus formation from the different explants

Callus formation rate was also low from nodes, internodes and leaves when IBA was used singly in the culture media in different concentration. Only 24.33 ± 1.52 callus

formation was recorded from the nodes, when IBA (4.0 mg/l) was used in the culture media, average day of callus initiation was 13.67 ± 0.09 and the callus were brownish green in colour. In case of internodes, percentage of callus formation was 28.24 ± 0.49 in same concentration of IBA. Average day of callus initiation was 13.25 ± 0.18 and colour of callus was yellowish green. Again IBA (4.0 mg/l) in the culture media showed 22.43 ± 1.13 leaves to form callus and in this case average day of callus initiation was 13.80 ± 0.30 and colour of callus was brownish green. The effects of different concentration of IBA on callus formation from the different explants are presented by histogram in Fig. 18.

3.3.1.3   Effects of NAA on callus formation from the different explants

Different concentration of NAA in the culture media showed medium callus formation rate from nodes, internodes and leaves. Percentage of callus formation was 27.21 ± 0.90 from the nodes when NAA (3.0 mg/l) was used in the culture media. Average day of callus initiation was 12.80 ± 0.65 and the callus were yellowish green in colour. Percentage of callus formation from internodes was 32.35 ± 1.15 in the same concentration of NAA and average day of callus initiation was 12.50 ± 0.30 and colour of callus was also yellowish green. In case of leaves, percentage of callus formation was recorded 25.87 ± 0.95 when NAA (3.0 mg/l) was used in the culture media. Average day of callus initiation was 13.10 ± 0.27 and colour of callus was yellowish green as callus of nodes and internodes. The effects of different concentration of NAA on callus formation from the different explants are presented by histogram in Fig. 19.

3.3.1.4   Effects of 2,4-D on callus formation from the different explants

Different concentration of 2,4-D in the culture media showed good result compared to other auxins (IAA, IBA, NAA) in callus formation from nodes, internodes and leaves. From nodes 38.56 ± 1.33 callus were formed when 2,4-D (2.0 mg/l) was used in the culture media. Average day of callus initiation was 13.10 ± 0.20 and colour of callus was light green. Percentage of callus formation from internodes was 42.71 ± 0.44 in the same

concentration of 2,4-D and this was the best result among different types of Auxins used individually in the culture media. Average day of callus initiation was 12.45 ± 0.28 and colour of callus was also light green. In case of leaves, percentage of callus formation was recorded 36.45 ± 0.63 when 2,4-D (2.0 mg/L) was used in the culture media. Average day of callus initiation was 13.10 ± 0.51 and colour of callus was yellowish green. The effects of different concentration of 2,4-D on callus formation from the different explants are presented by histogram in Fig. 20.

Table 9. Effects of different concentration of IAA, IBA, NAA and 2,4-D on callus formation from the different explants of *Stevia rebaudiana*.

Treatment (mg/l)		Average day of callus initiation			Percentage of explants induced callus			Colour of callus		
		*N	IN	L	N	IN	L	N	IN	L
IAA	1.0	14.60±0.89	13.6±0.44	14.75±0.71	10.57±1.18	10.13±1.82	8.46±0.39	Bg	Bg	Bg
	2.0	13.67±0.57	13.25±0.32	14.20±0.49	14.33±0.89	16.29±1.23	10.39±0.74	Bg	Bg	Bg
	3.0	13.33±0.53	13.25±0.82	13.55±0.06	17.25±1.42	20.34±0.80	15.12±1.70	Bg	Yg	Bg
	4.0	13.85±0.83	13.50±0.49	14.20±0.18	18.54±0.75	22.70±0.58	16.54±1.08	Bg	Yg	Bg
	5.0	14.40±0.66	13.80±0.60	14.67±0.35	16.76±1.43	18.41±0.57	13.37±0.46	Bg	Bg	Bg
IBA	1.0	14.45±0.39	13.60±0.38	14.70±0.47	10.69±1.63	12.44±1.32	10.66±0.58	Bg	Bg	Bg
	2.0	13.55±0.70	13.20±0.76	13.75±0.88	15.44±0.52	16.82±1.04	12.19±1.35	Bg	Bg	Bg
	3.0	13.10±0.35	12.85±0.07	13.25±0.75	20.47±0.38	24.86±1.19	18.35±1.05	Yg	Yg	Yg
	4.0	13.67±0.09	13.25±0.18	13.80±0.30	24.23±1.52	28.24±0.49	22.43±1.13	Bg	Yg	Bg
	5.0	14.15±0.92	13.40±0.27	14.33±0.18	18.15±1.20	20.11±0.38	15.76±0.80	Bg	Bg	Bg
NAA	1.0	14.15±0.94	13.45±0.42	14.33±0.37	16.68±0.78	20.59±0.44	14.05±0.69	Bg	Bg	Bg
	2.0	13.20±0.24	12.75±0.38	13.45±0.44	24.13±0.93	28.56±1.23	20.34±0.54	Yg	Yg	Bg
	3.0	12.80±0.65	12.50±0.30	13.10±0.27	27.21±0.90	32.35±1.15	25.87±0.95	Yg	Yg	Yg
	4.0	13.17±0.34	12.85±0.17	13.55±0.09	22.89±1.30	26.97±0.39	18.36±1.36	Yg	Yg	Bg
	5.0	13.85±0.77	13.00±0.65	14.00±0.15	20.45±1.08	22.85±1.51	16.49±1.02	Bg	Yg	Bg
2,4-D	1.0	13.80±0.17	13.20±0.33	14.00±0.28	25.12±0.46	28.22±0.60	24.13±1.72	Yg	Yg	Yg
	2.0	13.10±0.20	12.45±0.28	13.10±0.51	38.56±1.33	42.71±0.44	36.45±0.63	Lg	Lg	Yg
	3.0	12.67±0.59	12.20±0.34	12.85±0.22	37.78±0.79	40.64±0.85	35.74±0.77	Lg	Lg	Yg
	4.0	12.80±0.38	12.55±0.22	13.15±0.64	33.36±0.36	36.44±0.96	32.65±0.80	Yg	Yg	Yg
	5.0	13.55±0.51	12.70±0.37	13.80±0.47	28.53±0.98	30.82±1.48	25.09±0.62	Yg	Yg	Yg

*N=Nodes; IN= Internodes; L=Leaves, Gr = Green; Lg = Light Green; Yg = Yellowish Green; Bg = Brownish Green
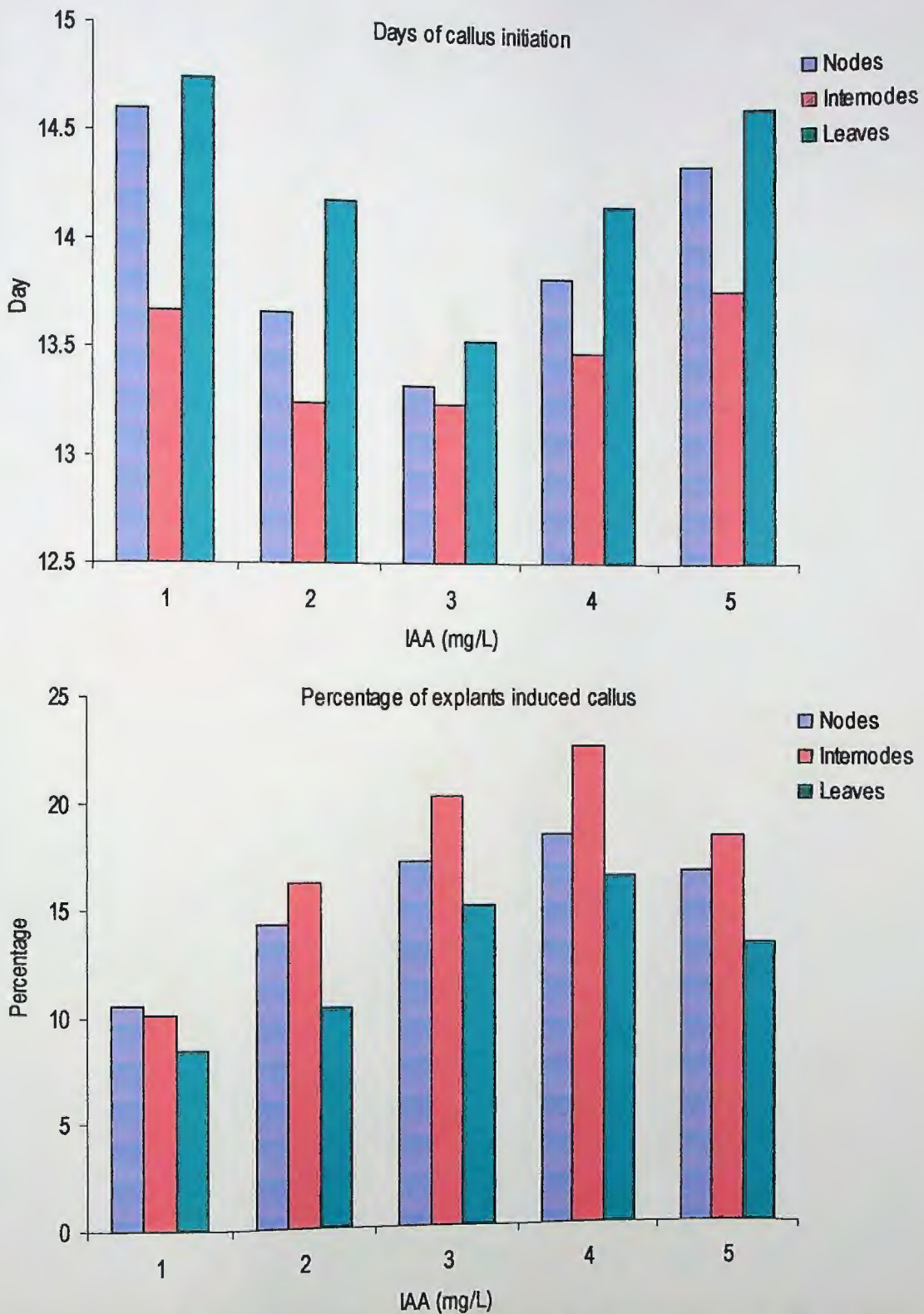
Fig. 17. Effects of different concentration of IAA on callus formation from the different explants of *Stevia rebaudiana*; days of callus initiation (upper), percentage of explants induced callus (lower).
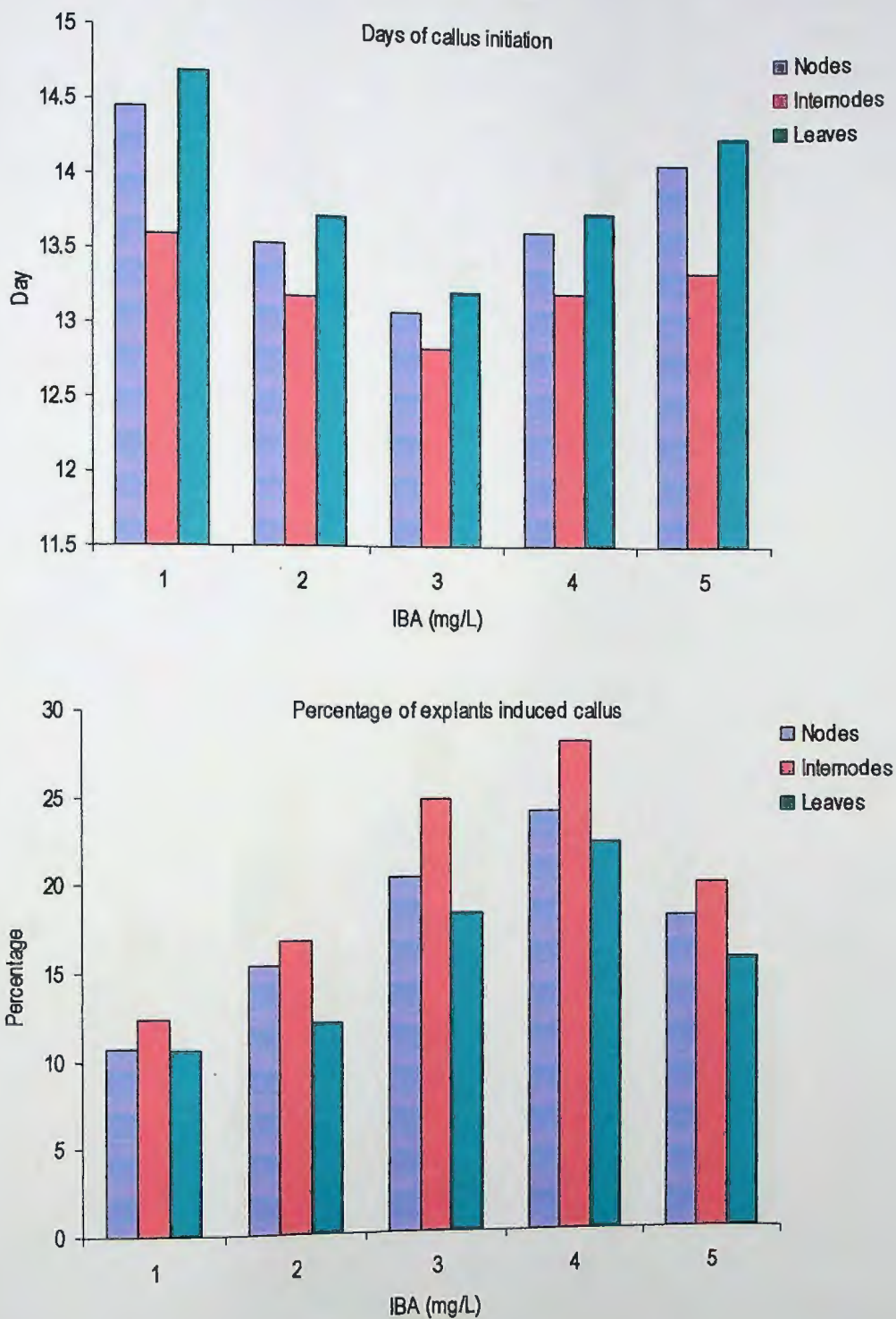
Fig. 18. Effects of different concentration of IBA on callus formation from the different explants of *Stevia rebaudiana*; days of callus initiation (upper), percentage of explants induced callus (lower).
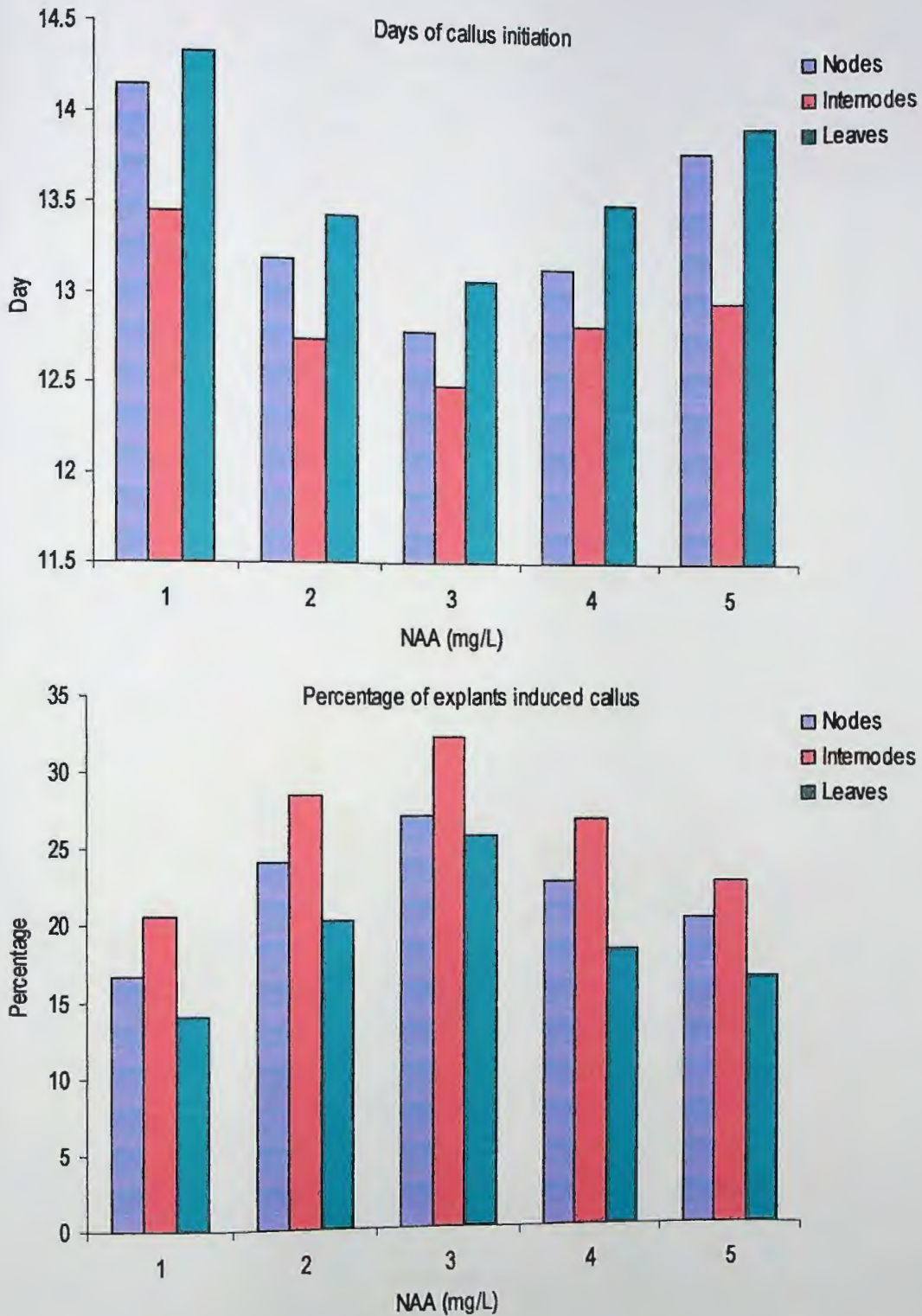
Fig. 19. Effects of different concentration of NAA on callus formation from the different explants of *Stevia rebaudiana*; days of callus initiation (upper), percentage of explants induced callus (lower).
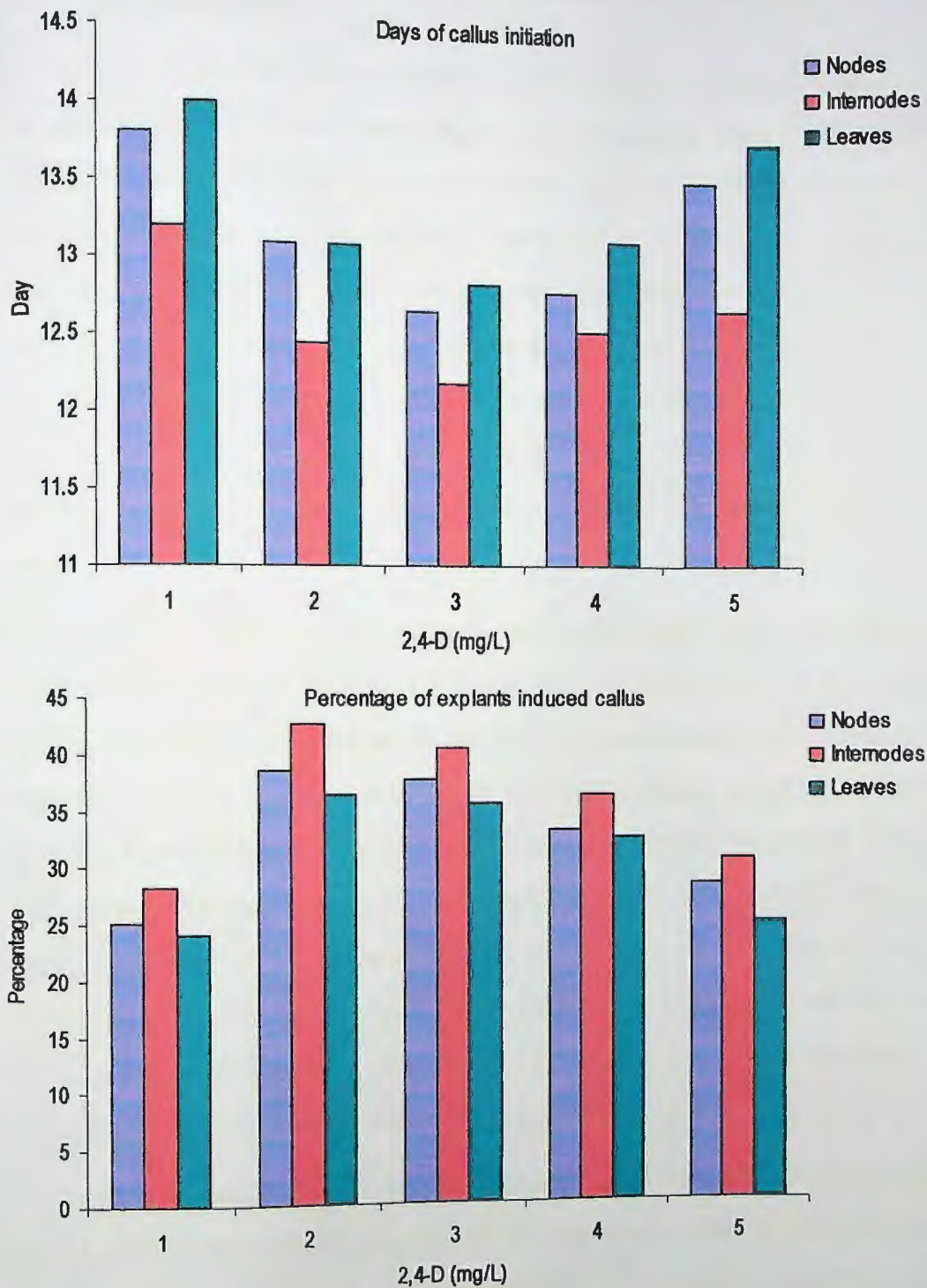
Fig. 20. Effects of different concentration of 2,4-D on callus formation from the different of *Stevia rebaudiana*; days of callus initiation (upper), percentage of explants induced callus (lower).

3.3.2 Effects of combined growth regulators on callus formation from the different explants

From the results of effects of individual growth regulators on callus formation from the different explants (3.3.1), it is clear that 2,4-D and NAA showed better result than IAA and IBA. To find out the effects of combined growth regulators on callus formation from nodes, internodes and leaves, five different concentrations of 2,4-D (1.0 mg/l, 1.5 mg/l, 2.0 mg/l, 2.5 mg/l, 3.0 mg/l) with five different concentrations of NAA (0.5 mg/l, 1.0 mg/l, 1.5 mg/l, 2.0 mg/l, 2.5 mg/l) were used in different combinations and the results are presented in Table 10. Percentages of callus formation were much better when 2,4-D and NAA were used in combination in the culture media compared to their individual effects on callus formation. In all cases NAA (1.5 mg/l)) with different concentration of 2,4-D showed the best result.

2,4-D (1.0 mg/l) with five different concentration of NAA: Highest percentage of callus formation was recorded 53.56 ± 0.53 from the nodes when 2,4-D (1.0 mg/l) + NAA (1.5 mg/l) was used in the culture media. Average day of callus initiation was 11.50 ± 0.13 and colour of callus was light green. In this combination, highest percentage of callus formation from internodes was 58.29 ± 0.38, average day of callus initiation was 11.15 ± 0.25 and the callus were light green in colour. Again highest percentage of callus formation was recorded 49.55 ± 1.21 in the same combination of 2,4-D and NAA when the leaves were used as explants. Average day of callus initiation was 12.20 ± 0.30 and colour of callus was also light green. The effects of 2,4-D (1.0 mg/l) with different concentration of NAA on callus formation from the different explants are presented by histogram in Fig. 21.

2,4-D (1.5 mg/l) with five different concentration of NAA: When 2,4-D (1.5 mg/l) + NAA (1.5 mg/l) was used in the culture media, highest percentage of callus formation observed 60.44 ± 1.26 from the nodes. Average day of callus initiation was 11.50 ± 0.17 and colour of callus was green. Highest percentage of callus formation was 65.10 ± 0.08 in case of internodes in the same combination and average day of callus initiation was 11.25 ±

0.46 and the callus were green in colour. Again highest percentage of callus formation was 57.08 ± 0.67 from the leaves in the same combination of 2,4-D and IAA. Average day of callus initiation was 11.80 ± 0.16 and colour of callus was light green. The effects of 2,4-D (1.5 mg/l) with different concentration of NAA on callus formation from the different explants are presented by histogram in Fig. 22.

2,4-D (2.0 mg/l) with five different concentration of NAA: In case of 2,4-D (2.0 mg/l) + NAA (1.5 mg/l) in the culture media, highest percentage of callus formation from nodes was recorded 66.81 ± 0.85, average day of callus initiation was 11.85 ± 0.16 and colour of callus was green. In same combination highest percentage of callus formation from the internodes was 74.62 ± 1.06. Average day of callus initiation was 10.50 ± 0.20 and the callus were green in colour. Again leaves as explants, highest percentage of callus formation was 65.59 ± 0.90 in same combination of 2,4-D with NAA. Average day of callus initiation was 11.50 ± 0.35 and colour of callus was also green. The effects of 2,4-D (2.0 mg/l) with different concentration of NAA on callus formation from the different explants are presented by histogram in Fig. 23.

2,4-D (2.5 mg/l) with five different concentration of NAA: 2,4-D (2.5 mg/l) + NAA (1.5 mg/l) was found the most effective combination for callus formation in the culture media. All types of explants (nodes, internodes, leaves) showed highest percentage of callus formation in this combination and all callus were green in colour. Highest percentage of callus formation was 70.34 ± 0.39 when the nodes were used as explants, average day of callus initiation was 10.50 ± 0.15. But in case of internodes highest percentage of callus formation was 82.69 ± 1.40 which was the best result of callus formation. Average day of callus initiation was 10.00 ± 0.05. Again in this combination the leaves showed highest percentage of callus formation which was 68.21 ± 0.87, average day of callus formation was 11.00 ± 0.15. The effects of 2,4-D (2.5 mg/L) with different concentration of NAA on callus formation from the different explants are presented by histogram in Fig. 24.

2,4-D (3.0 mg/l) with five different concentration of NAA: Highest percentage of callus formation was recorded 55.24 ± 1.18 from the nodes when 2,4-D (3.0 mg/l) + NAA (1.5 mg/l) was used in the culture media. Average day of callus initiation was 12.33 ± 0.17 and colour of callus was light green. In this combination, highest percentage of callus formation from internodes was 62.50 ± 0.63, average day of callus initiation was 12.25 ± 0.30 and the callus were green in colour. Again highest percentage of callus formation was 52.81 ± 0.52 in the same combination of 2,4-D and NAA when the leaves were used as explants. Average day of callus initiation was 12.45 ± 0.29 and colour of callus was light green. The effects of 2,4-D (3.0 mg/l) with different concentration of NAA on callus formation from the different explants are presented by histogram in Fig. 25.

Table 10. Effects of different concentration of 2,4-D with different concentration of NAA on callus formation from the different explants of *Stevia rebaudiana*.

Treatment (mg/l)		Average day of callus initiation			Percentage of explants induced callus			Colour of callus		
2,4-D	NAA	*N	IN	L	N	IN	L	N	IN	L
1.0	0.5	13.25±0.35	12.80±0.18	13.75±0.33	35.60±1.23	38.22±1.59	32.58±1.06	Yg	Yg	Yg
	1.0	12.10±0.08	11.50±0.40	12.50±0.17	48.89±0.87	52.57±0.80	44.75±1.54	Lg	Lg	Lg
	1.5	11.50±0.13	11.15±0.25	12.20±0.30	53.56±0.53	58.29±0.38	49.55±1.21	Lg	Lg	Lg
	2.0	12.50±0.26	12.10±0.39	12.85±0.22	40.12±0.49	45.80±1.55	38.43±0.76	Yg	Yg	Yg
	2.5	13.33±0.35	13.00±0.20	14.33±0.45	33.08±0.79	35.51±1.19	30.67±0.92	Bg	Yg	Bg
1.5	0.5	14.00±0.04	13.25±0.33	13.75±0.28	41.62±1.04	44.03±0.55	38.33±0.87	Yg	Yg	Bg
	1.0	13.00±0.12	12.50±0.25	13.00±0.50	52.59±0.36	55.78±0.32	48.29±0.59	Lg	Lg	Yg
	1.5	11.50±0.17	11.25±0.46	11.80±0.16	60.44±1.26	65.10±0.08	57.08±0.67	Gr	Gr	Lg
	2.0	12.20±0.09	11.67±0.18	12.50±0.28	46.57±1.33	51.93±0.95	44.12±1.36	Yg	Lg	Yg
	2.5	13.20±0.23	12.40±0.39	13.50±0.43	42.83±0.48	46.46±0.78	39.50±1.08	Yg	Yg	Bg
2.0	0.5	13.10±0.15	12.50±0.50	13.25±0.14	45.22±0.39	48.50±1.35	40.28±1.28	Yg	Yg	Yg
	1.0	12.20±0.03	12.00±0.35	12.50±0.33	55.30±0.28	58.11±0.86	52.47±0.73	Lg	Lg	Lg
	1.5	11.85±0.16	10.50±0.20	11.50±0.35	66.81±0.85	74.62±1.06	65.59±0.90	Gr	Gr	Gr
	2.0	11.80±0.23	11.50±0.55	12.00±0.48	50.34±1.17	55.04±0.36	48.34±1.43	Lg	Lg	Lg
	2.5	12.50±0.37	12.50±0.15	13.00±0.33	48.91±1.02	52.59±0.77	45.76±0.83	Yg	Lg	Yg
2.5	0.5	12.50±0.02	12.00±0.12	13.00±0.10	52.27±0.71	58.23±0.45	48.22±0.59	Lg	Lg	Lg
	1.0	11.25±0.08	10.80±0.20	11.50±0.16	64.59±0.44	70.44±1.88	61.40±1.19	Gr	Gr	Lg
	1.5	10.50±0.15	10.00±0.05	11.00±0.15	70.34±0.39	82.69±1.40	68.21±0.87	Gr	Gr	Gr
	2.0	11.20±0.08	10.65±0.20	11.40±0.06	62.35±0.90	71.82±0.91	58.70±0.61	Gr	Gr	Lg
	2.5	12.25±0.19	12.00±0.05	12.50±0.15	58.86±0.57	68.07±1.11	54.72±1.20	Lg	Gr	Lg
3.0	0.5	14.10±0.11	13.33±0.36	14.25±0.31	38.25±1.68	42.78±0.65	35.66±1.09	Bg	Yg	Bg
	1.0	13.16±0.23	12.75±0.45	13.20±0.66	49.60±0.45	54.33±0.89	45.92±1.14	Yg	Lg	Lg
	1.5	12.33±0.17	12.25±0.30	12.45±0.29	55.24±1.18	62.50±0.63	52.81±0.52	Lg	Gr	Lg
	2.0	12.67±0.08	12.40±0.22	12.85±0.31	43.58±1.70	47.86±0.72	40.54±0.88	Lg	Yg	Yg
	2.5	13.40±0.23	13.00±0.15	13.67±0.47	36.14±1.98	39.25±0.34	32.56±1.30	Bg	Bg	Bg

*N=Nodes; IN= Internodes; L=Leaves; Gr = Green; Lg = Light Green; Yg = Yellowish Green; Bg = Brownish Green

Fig. 21. Effects of 2,4-D (1.0 mg/l) with different concentration of NAA on callus formation from the different explants of *Stevia rebaudiana*; days of callus initiation (upper), percentage of explants induced callus (lower).

Fig. 22. Effects of 2,4-D (1.5 mg/l) with different concentration of NAA on callus formation from the different explants of *Stevia rebaudiana*; days of callus initiation (upper), percentage of explants induced callus (lower).
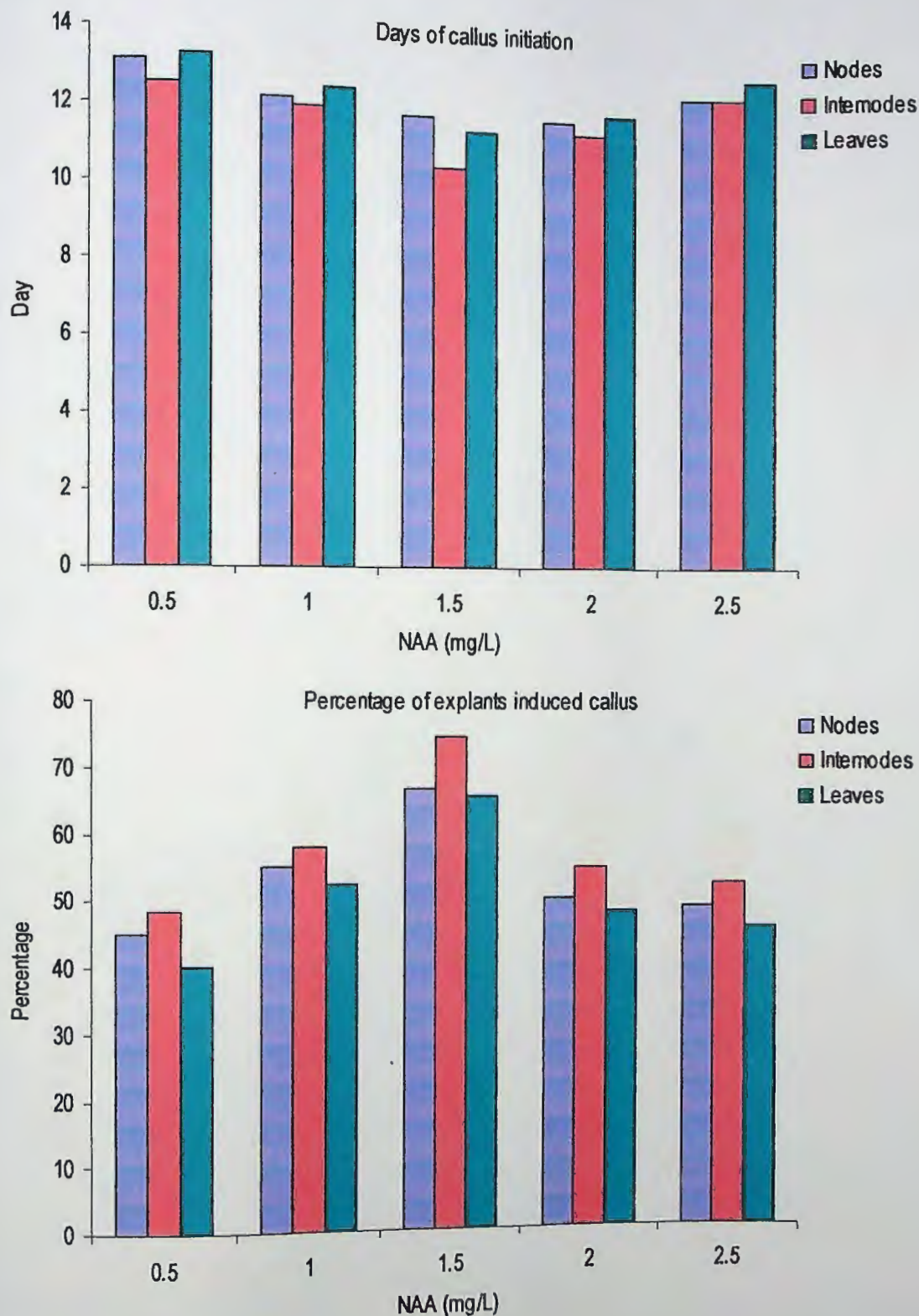
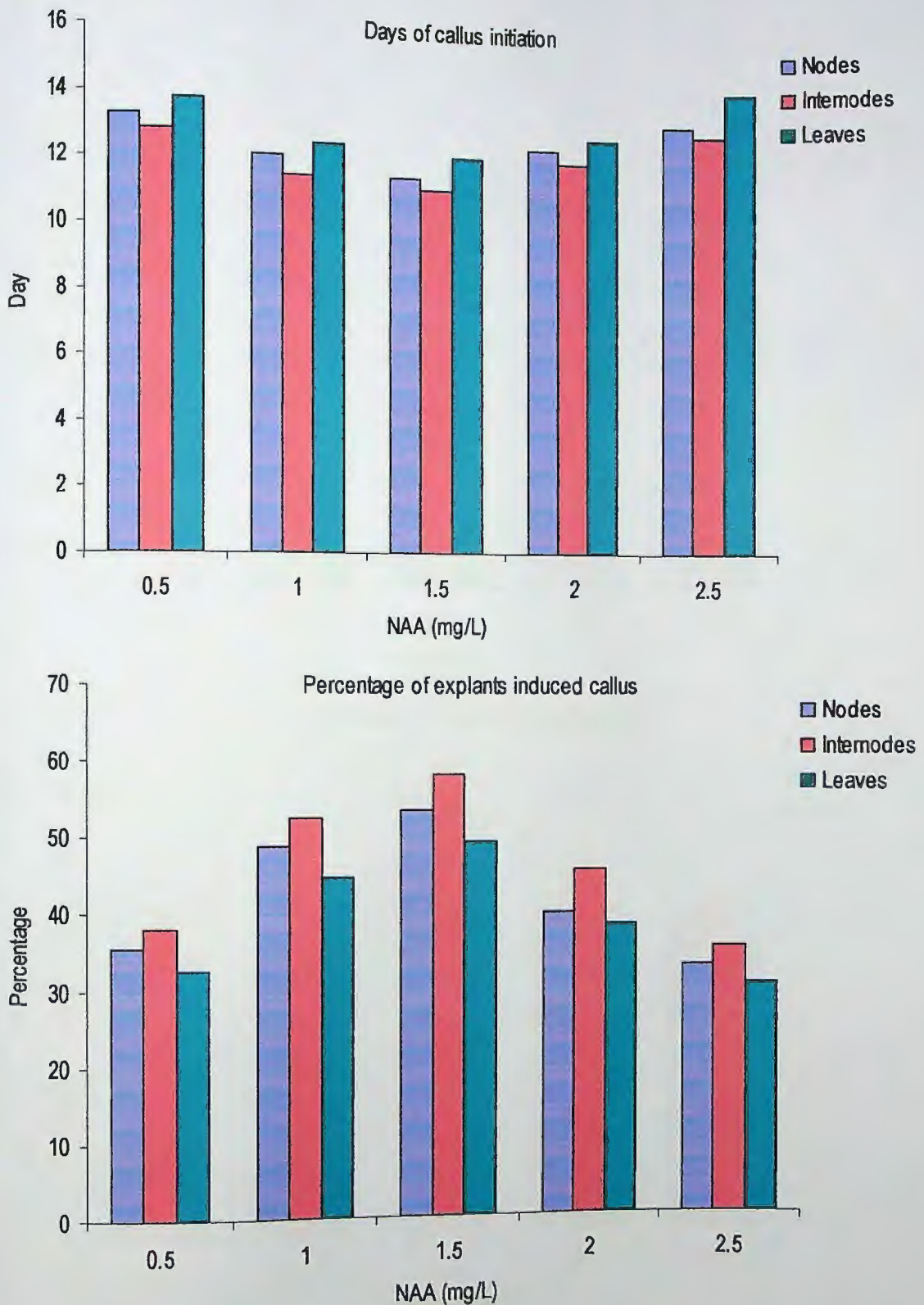**Fig. 23.** Effects of 2,4-D (2.0 mg/l) with different concentration of NAA on callus formation from the different explants of *Stevia rebaudiana*; days of callus initiation (upper), percentage of explants induced callus (lower).
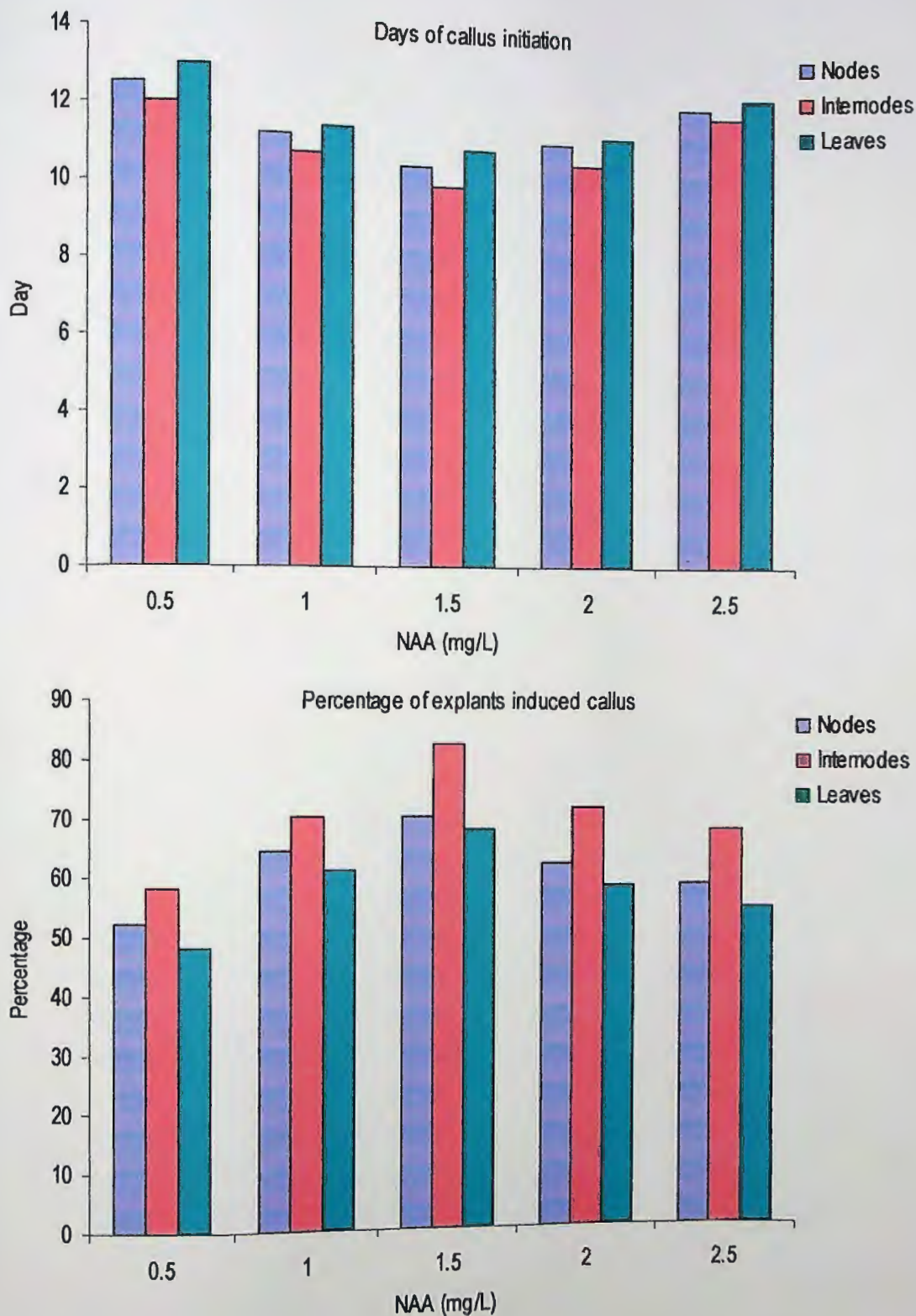
Fig. 24. Effects of 2,4-D (2.5 mg/l) with different concentration of NAA on callus formation from the different explants of *Stevia rebaudiana*; days of callus initiation (upper), percentage of explants induced callus (lower).
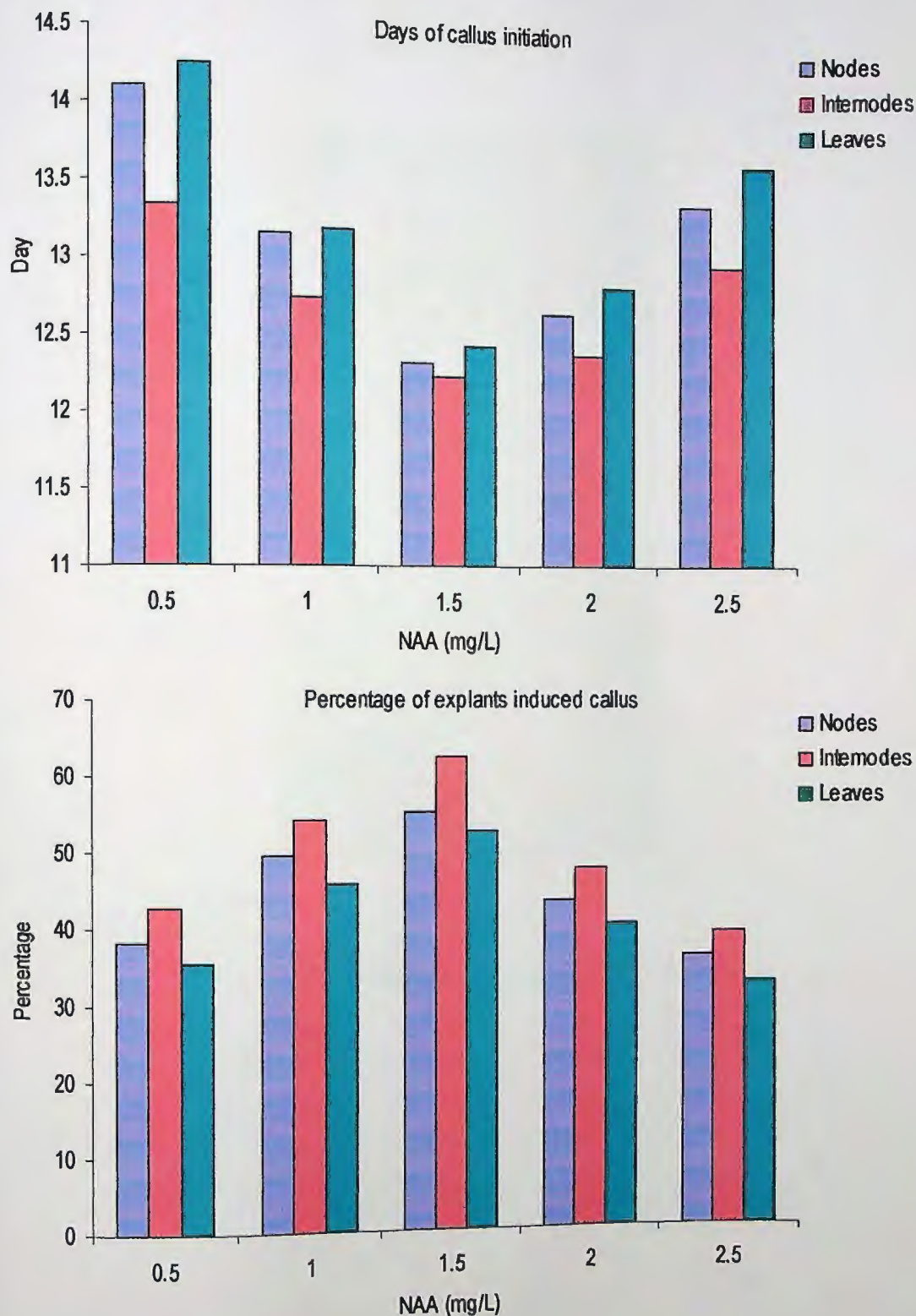
Fig. 25. Effects of 2,4-D (3.0 mg/l) with different concentration of NAA on callus formation from the different explants of *Stevia rebaudiana*; days of callus initiation (upper), percentage of explants induced callus (lower).
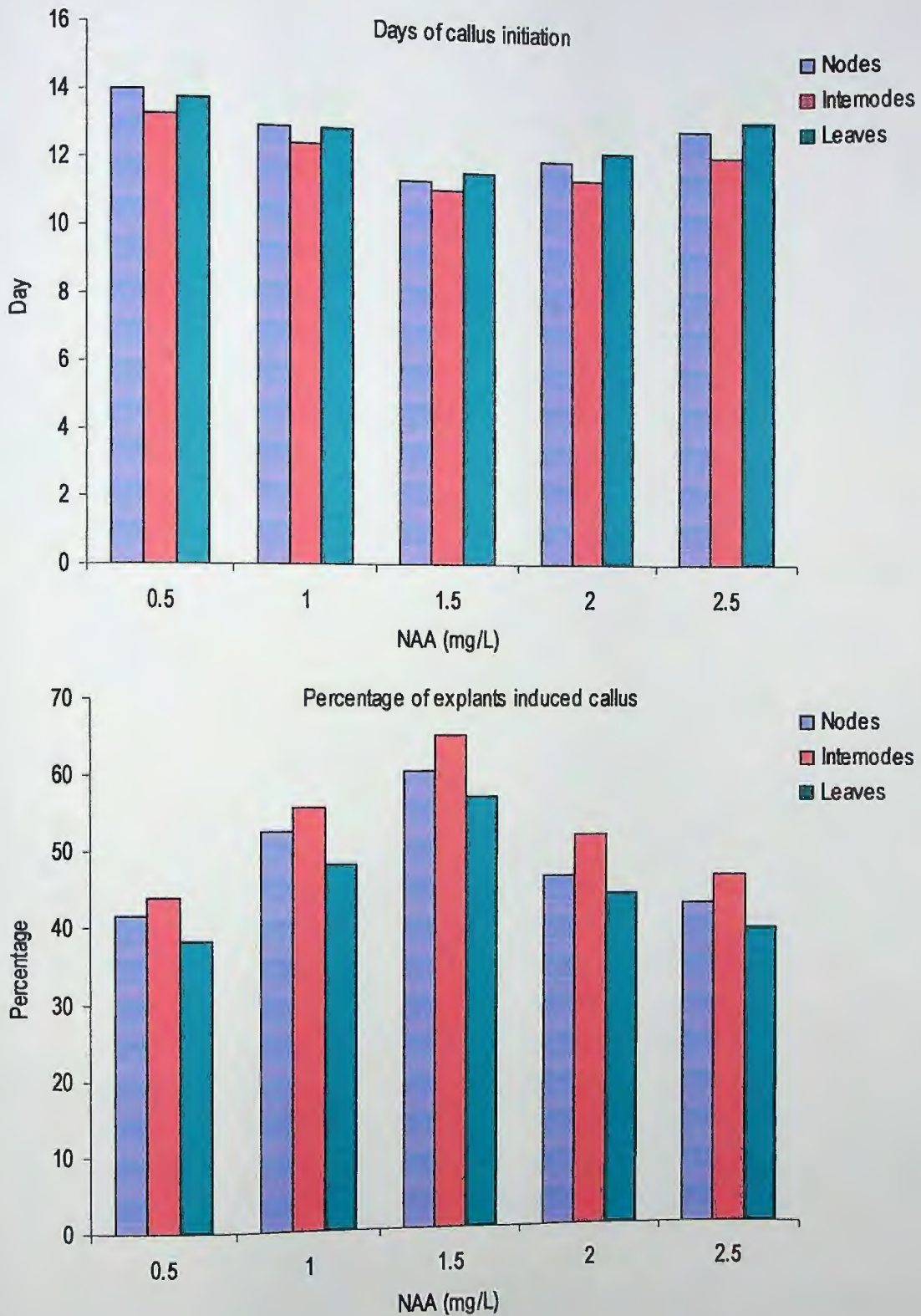
Plate 3. Callus formation from explant of *Stevia rebaudiana*

3.4 Indirect shoot proliferation from callus of the different explants

Different concentration of Cytokinins and Auxins were used in the culture media to observe the effects of the growth regulators on indirect shoot proliferation from the callus of nodes, internodes and leaves. In all cases callus of nodes showed the highest percentage of proliferated shoots compared to the callus of internodes and leaves.

3.4.1 Effects of individual growth regulators on indirect shoot proliferation from callus of the different explants

By comparing the results of direct shoot proliferation from different explants (3.2) it is clear that combination of different growth regulators (Cytokinins + Auxins) in the culture media was more effective on direct shoot proliferation compared to individual growth regulators. For this reason in case of indirect shoot proliferation from callus of different explants only effects of combined growth regulators were observed.

3.4.2 Effects of combined growth regulators on indirect shoot proliferation from callus of the different explants

To find out the effects of combined growth regulators on indirect shoot proliferation from the callus of nodes, internodes and leaves, different concentration of Cytokinins (BAP) with different concentration of Auxins (IAA, IBA, NAA) were used in different combinations and combination of BAP and NAA in the culture media was found the most effective on indirect shoot proliferation from callus of the different explants.

3.4.2.1 Effects of different concentration of BAP with different concentration of IAA

Four different concentrations of BAP (1.0 mg/l, 1.5 mg/l, 2.0 mg/l, 2.5 mg/l) with five different concentrations of IAA (0.1 mg/l, 0.2 mg/l, 0.3 mg/l, 0.4 mg/l, 0.5 mg/l) were used in the culture media to find out the combined effects of BAP and IAA on indirect shoots proliferation from the callus of nodes, internodes and leaves and the results are presented in Table 11. In most of the cases IAA (0.2 mg/l) or IAA (0.3 mg/l) with different concentration of BAP showed the best result.

BAP (1.0 mg/l) with five different concentration of IAA: In case of callus from nodes the highest percentage of proliferated shoots was observed when BAP (1.0 mg/l) + IAA (0.3 mg/l) was used in the culture media which was 66.17 ± 0.62. In this combination average number of shoots per callus was 3.25 ± 0.65 and average length of the longest shoots was 2.40 ± 0.16 cm. But in case of callus of internodes, the highest percentage of proliferated shoots was 63.18 ± 0.45 in same combination of the growth regulators and average number of shoots per callus was 3.30 ± 0.22 and average length of the longest shoots was 2.46 ± 0.55 cm. Again in the same combination of BAP and IAA the highest percentage of proliferated shoots from the callus of leaves was 61.58 ± 1.38, average number of shoots per callus was 3.33 ± 0.26 and average length of the longest shoots was 2.49 ± 0.20 cm. Other combinations showed average performances of shoot proliferation of the callus. The effects of BAP (1.0 mg/l) with different concentration of IAA on indirect shoot proliferation from callus of the different explants are presented by histogram in Fig. 26.

BAP (1.5 mg/l) with five different concentration of IAA: When BAP (1.5 mg/l) + IAA (0.2 mg/l) was used in the culture media, highest percentage of proliferated shoots was recorded from the callus of nodes which was 70.22 ± 1.21, average number of shoots per callus was 3.45 ± 0.39 and average length of the longest shoots was 2.42 ± 0.13 cm. But when BAP (1.5 mg/l) + IAA (0.3 mg/l) was used, the highest percentage of proliferated shoots from the callus of internodes was 67.48 ± 0.17 and average number of shoots per callus was 3.35 ± 0.59 and average length of the longest shoots was 2.55 ± 0.28 cm. Again in case of callus from leaves, the highest percentage of proliferated shoots was 65.17 ± 0.23 in same combination of the growth regulators and average number of shoots per callus was 3.40 ± 0.63 and average length of the longest shoots was 2.53 ± 0.31 cm. Other combinations showed average performances of shoot proliferation of the callus. The effects of BAP (1.5 mg/l) with different concentration of IAA on indirect shoot proliferation from callus of the different explants are presented by histogram in Fig. 27.

BAP (2.0 mg/l) with five different concentration of IAA: BAP (2.0 mg/l) + IAA (0.2 mg/l) in the culture media showed the highest percentage of proliferated shoots from the callus of nodes which was 75.62 ± 0.84. This is the best result among the BAP + IAA combinations. In this combination average number of shoots per callus was 3.80 ± 0.17 and average length of the longest shoots was 2.57 ± 0.09 cm. In the same combination of the growth regulators highest percentage of proliferated shoots from the callus of internodes was 73.67 ± 1.07, average number of shoots per callus was 3.85 ± 0.11 and average length of the longest shoots was 2.69 ± 0.23 cm. But in case of callus from leaves, the highest percentage of proliferated shoots was observed in the same combination of BAP and IAA in the culture media which was 72.42 ± 0.76. In this combination average number of shoots per callus was 3.45 ± 0.82 and average length of the longest shoots was 2.64 ± 0.12 cm. Other combinations showed average performances of shoot proliferation of the callus. The effects of BAP (2.0 mg/l) with different concentration of IAA on indirect shoot proliferation from callus of the different explants are presented by histogram in Fig. 28.

BAP (2.5 mg/l) with five different concentration of IAA: Highest percentage of proliferated shoots was found from the callus of nodes which was 70.86 ± 1.05 when BAP (2.5 mg/l) + IAA (0.2 mg/l) was used in the culture media and average number of shoots per callus was 3.40 ± 0.11 and average length of the longest shoots was 2.51 ± 0.20 cm. In same combination of the growth regulators highest percentage of proliferated shoots from the callus of internodes was 69.75 ± 1.29, average number of shoots per callus was 3.40 ± 0.31 and average length of the longest shoots was 2.60 ± 0.51 cm. In case of callus from leaves, highest percentage of proliferated shoots was 67.54 ± 0.95 in same combination of the growth regulators and average number of shoots per callus was 3.33 ± 0.43 and average length of the longest shoots was 2.53 ± 0.28 cm. Other combinations showed average performances of shoot proliferation of the callus. The effects of BAP (2.5 mg/l) with different concentration of IAA on indirect shoot proliferation from callus of the different explants are presented by histogram in Fig. 29.
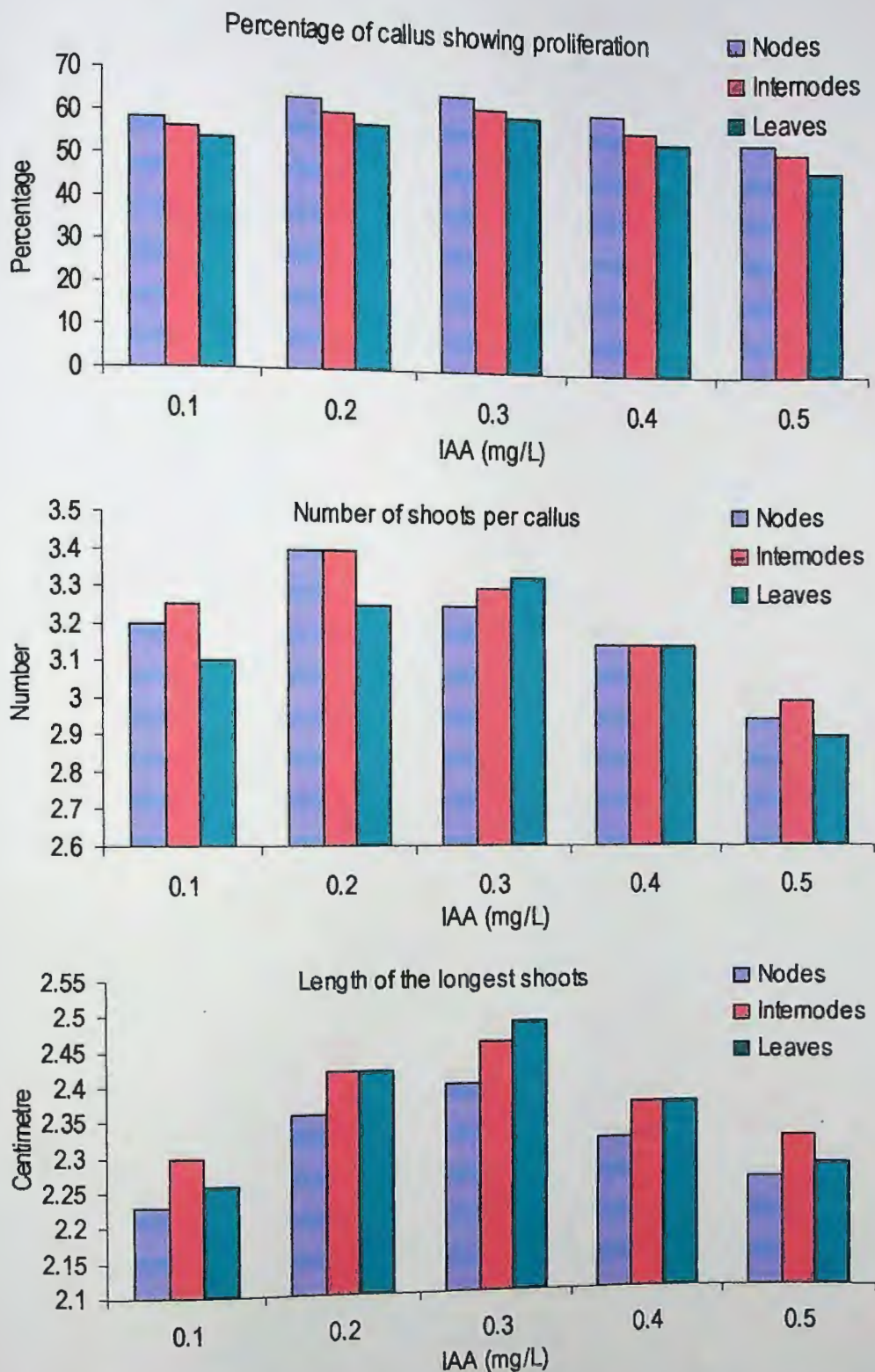
Table 11. Effects of different concentration of BAP with different concentration of IAA on indirect shoot proliferation from callus of the different explants of *Stevia rebaudiana*.

Treatment (mg/l)		Percentage of callus showing proliferation			Average number of shoots per callus			Average length of the longest shoots (cm)		
BAP	IAA	*N	IN	L	N	IN	L	N	IN	L
1.0	0.1	58.30±0.73	56.40±1.90	53.85±1.13	3.20±0.38	3.25±0.81	3.10±0.07	2.23±0.22	2.30±0.41	2.26±0.30
	0.2	63.84±0.89	60.72±0.37	58.26±0.28	3.40±0.14	3.40±0.69	3.25±0.17	2.36±0.10	2.42±0.28	2.42±0.12
	0.3	66.17±0.62	63.18±0.45	61.58±1.38	3.25±0.65	3.30±0.22	3.33±0.26	2.40±0.16	2.46±0.55	2.49±0.20
	0.4	62.75±1.15	58.67±0.88	56.42±0.49	3.15±0.29	3.15±0.97	3.15±0.81	2.32±0.31	2.37±0.20	2.37±0.41
	0.5	56.42±0.34	53.82±0.76	49.60±0.93	2.95±0.08	3.00±0.66	2.90±0.30	2.26±0.24	2.32±0.77	2.28±0.35
1.5	0.1	64.58±1.06	62.33±1.58	58.47±1.22	3.30±0.25	3.30±0.40	3.15±0.13	2.30±0.06	2.37±0.32	2.33±0.15
	0.2	70.22±1.21	65.24±0.49	62.55±0.56	3.45±0.39	3.45±0.37	3.33±0.49	2.42±0.13	2.52±0.11	2.46±0.36
	0.3	68.45±0.57	67.48±0.17	65.17±0.23	3.35±0.60	3.35±0.59	3.40±0.63	2.47±0.52	2.55±0.28	2.53±0.31
	0.4	63.84±0.43	62.65±1.33	59.75±0.38	3.30±0.22	3.25±0.18	3.25±0.75	2.38±0.34	2.45±0.26	2.41±0.20
	0.5	61.33±0.29	60.42±0.86	52.20±1.27	3.20±0.62	3.15±0.54	3.10±0.39	2.33±0.10	2.39±0.88	2.35±0.14
2.0	0.1	69.20±0.77	68.15±0.69	66.58±0.80	3.60±0.48	3.65±0.79	3.33±0.92	2.36±0.14	2.43±0.09	2.48±0.44
	0.2	75.62±0.84	73.67±1.07	72.42±0.76	3.80±0.17	3.85±0.11	3.45±0.82	2.57±0.09	2.69±0.23	2.64±0.12
	0.3	71.44±0.70	70.25±0.46	68.33±1.58	3.65±0.55	3.70±0.23	3.75±0.48	2.51±0.25	2.60±0.19	2.56±0.29
	0.4	67.15±1.32	66.45±0.94	63.78±0.49	3.60±0.20	3.60±0.61	3.40±0.06	2.46±0.62	2.55±0.44	2.44±0.51
	0.5	63.32±1.64	62.84±1.26	58.67±0.60	3.50±0.42	3.45±0.25	3.25±0.44	2.38±0.57	2.46±0.06	2.39±0.27
2.5	0.1	63.25±0.44	61.45±0.58	60.15±1.24	3.25±0.46	3.30±0.68	3.15±0.80	2.32±0.29	2.41±0.82	2.45±0.17
	0.2	70.86±1.05	69.75±1.29	67.54±0.95	3.40±0.11	3.40±0.31	3.33±0.43	2.51±0.20	2.60±0.51	2.53±0.28
	0.3	65.50±0.39	64.28±0.44	63.47±0.68	3.30±0.29	3.35±0.53	3.45±0.27	2.42±0.14	2.53±0.12	2.44±0.08
	0.4	60.88±0.51	60.17±0.92	59.20±1.04	3.20±0.71	3.20±0.17	3.25±0.31	2.34±0.08	2.39±0.43	2.37±0.25
	0.5	57.62±0.37	57.84±0.85	54.43±1.40	3.15±0.28	3.10±0.22	3.00±0.56	2.27±0.13	2.34±0.31	2.32±0.60

*N=Nodes; IN= Internodes; L=Leaves

Fig. 26. Effects of BAP (1.0 mg/l) with different concentration of IAA on indirect shoot proliferation from callus of the different explants of *Stevia rebaudiana*; percentage proliferation (upper), number of shoots per callus (middle), length of longest shoot (lower).

Fig. 27. Effects of BAP (1.5 mg/l) with different concentration of IAA on indirect shoot proliferation from callus of the different explants of *Stevia rebaudiana*; percentage proliferation (upper), number of shoots per callus (middle), length of longest shoot (lower).

Fig. 28. Effects of BAP (2.0 mg/l) with different concentration of IAA on indirect shoot proliferation from callus of the different explants of *Stevia rebaudiana*; percentage proliferation (upper), number of shoots per callus (middle), length of longest shoot (lower).

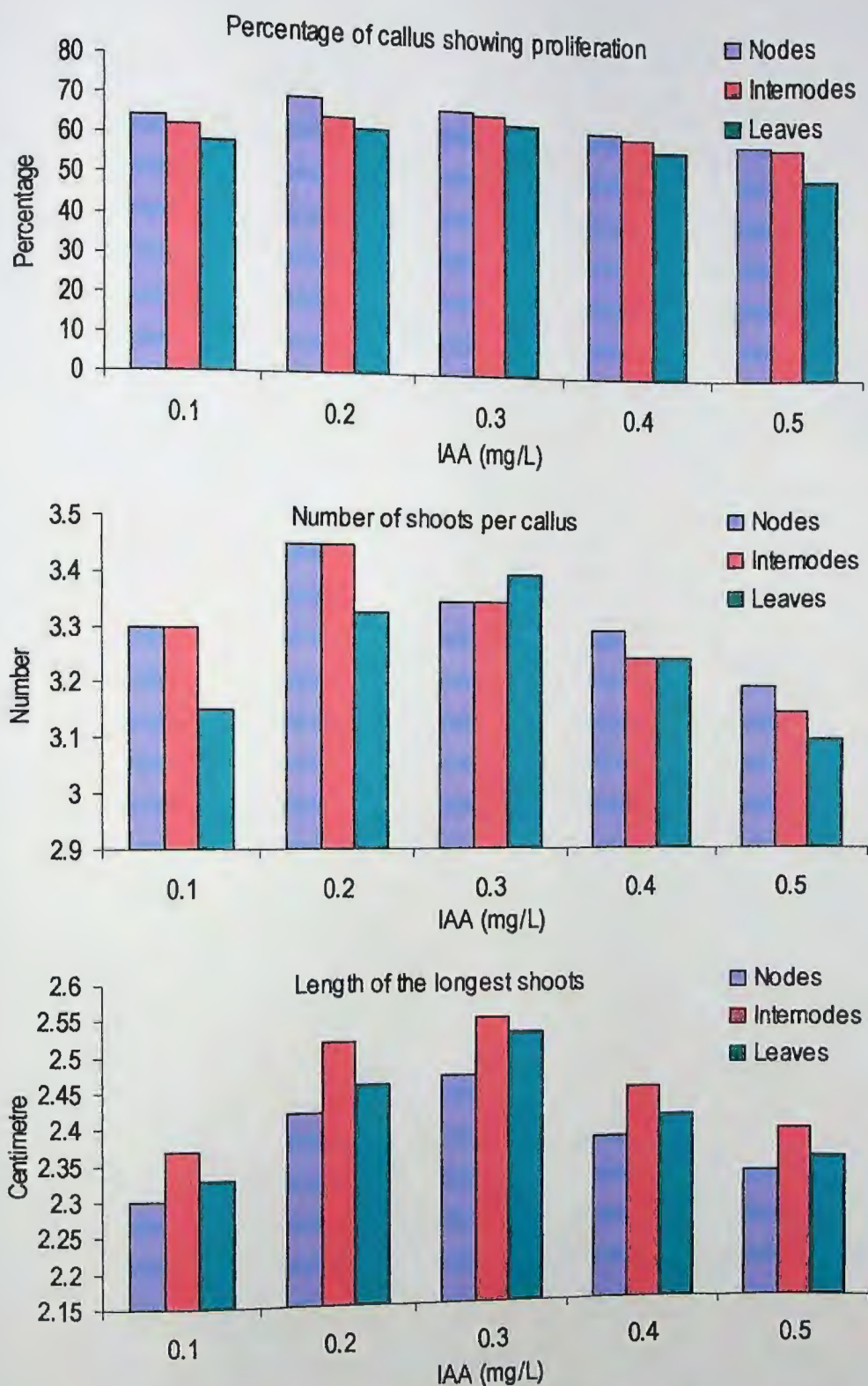**Fig. 29.** Effects of BAP (2.5 mg/l) with different concentration of IAA on indirect shoot proliferation from callus of the different explants of *Stevia rebaudiana*; percentage proliferation (upper), number of shoots per callus (middle), length of longest shoot (lower).

3.4.2.2 Effects of different concentration of BAP with different concentration of IBA

Four different concentrations of BAP (1.0 mg/l, 1.5 mg/l, 2.0 mg/l, 2.5 mg/l) with five different concentrations of IBA (0.1 mg/l, 0.2 mg/l, 0.3 mg/l, 0.4 mg/l, 0.5 mg/l) were used in the culture media to find out the combined effects of BAP and IBA on indirect shoots proliferation from the callus of nodes, internodes and leaves and the results are presented in Table 12. In most of the cases IBA (0.2 mg/l) or IBA (0.3 mg /l) with different concentration of BAP showed the best result.

BAP (1.0 mg/l) with five different concentration of IBA: In case of callus from nodes the highest percentage of proliferated shoots was observed when BAP (1.0 mg/l) + IBA (0.3 mg/l) was used in the culture media which was 69.24 ± 0.81. In this combination average number of shoots per callus was 3.40 ± 0.07 and average length of the longest shoots was 2.57 ± 0.40 cm. But in case of callus of internodes, the highest percentage of proliferated shoots was 65.17 ± 1.19 in same combination of the growth regulators and average number of shoots per callus was 3.20 ± 0.48 and average length of the longest shoots was 2.69 ± 0.27 cm. Again in the same combination of BAP and IBA the highest percentage of proliferated shoots from the callus of leaves was 62.33 ± 0.58, average number of shoots per callus was 3.45 ± 0.12 and average length of the longest shoots was 2.49 ± 0.36 cm. Other combinations showed average performances of shoot proliferation of the callus. The effects of BAP (1.0 mg/l) with different concentration of IBA on indirect shoot proliferation from callus of the different explants are presented by histogram in Fig. 30.

BAP (1.5 mg/l) with five different concentration of IBA: When BAP (1.5 mg/l) + IBA (0.2 mg/l) was used in the culture media, highest percentage of proliferated shoots was recorded from the callus of nodes which was 70.24 ± 1.39, average number of shoots per callus was 3.55 ± 0.41 and average length of the longest shoots was 2.51 ± 0.33 cm. In the same combination if BAP and IBA the highest percentage of proliferated shoots from the callus of internodes was recorded 68.33 ± 0.13 and average number of shoots per callus

was 3.45 ± 0.50 and average length of the longest shoots was 2.65 ± 0.28 cm. But when BAP (1.5 mg/l) + IBA (0.3 mg/l) was used in the culture media, the highest percentage of proliferated shoots from callus of leaves was recorded 67.60 ± 0.58 and average number of shoots per callus was 3.55 ± 0.24 and average length of the longest shoots was 2.62 ± 0.17 cm. Other combinations showed average performances of shoot proliferation of the callus. The effects of BAP (1.5 mg/l) with different concentration of IBA on indirect shoot proliferation from callus of the different explants are presented by histogram in Fig. 31.

BAP (2.0 mg/l) with five different concentration of IBA: BAP (2.0 mg/l) + IBA (0.2 mg/l) in the culture media showed the highest percentage of proliferated shoots from the callus of nodes which was 78.33 ± 0.24. This is the best result among the BAP + IBA combinations. In this combination average number of shoots per callus was 4.05 ± 0.31 and average length of the longest shoots was 2.61 ± 0.06 cm. In the same combination of the growth regulators highest percentage of proliferated shoots from the callus of internodes was 75.43 ± 0.80, average number of shoots per callus was 3.75 ± 0.26 and average length of the longest shoots was 2.72 ± 0.49 cm. But in case of callus from leaves, the highest percentage of proliferated shoots was observed in the same combination of BAP and IBA in the culture media which was 71.43 ± 0.53. In this combination average number of shoots per callus was 3.55 ± 0.18 and average length of the longest shoots was 2.60 ± 0.22 cm. Other combin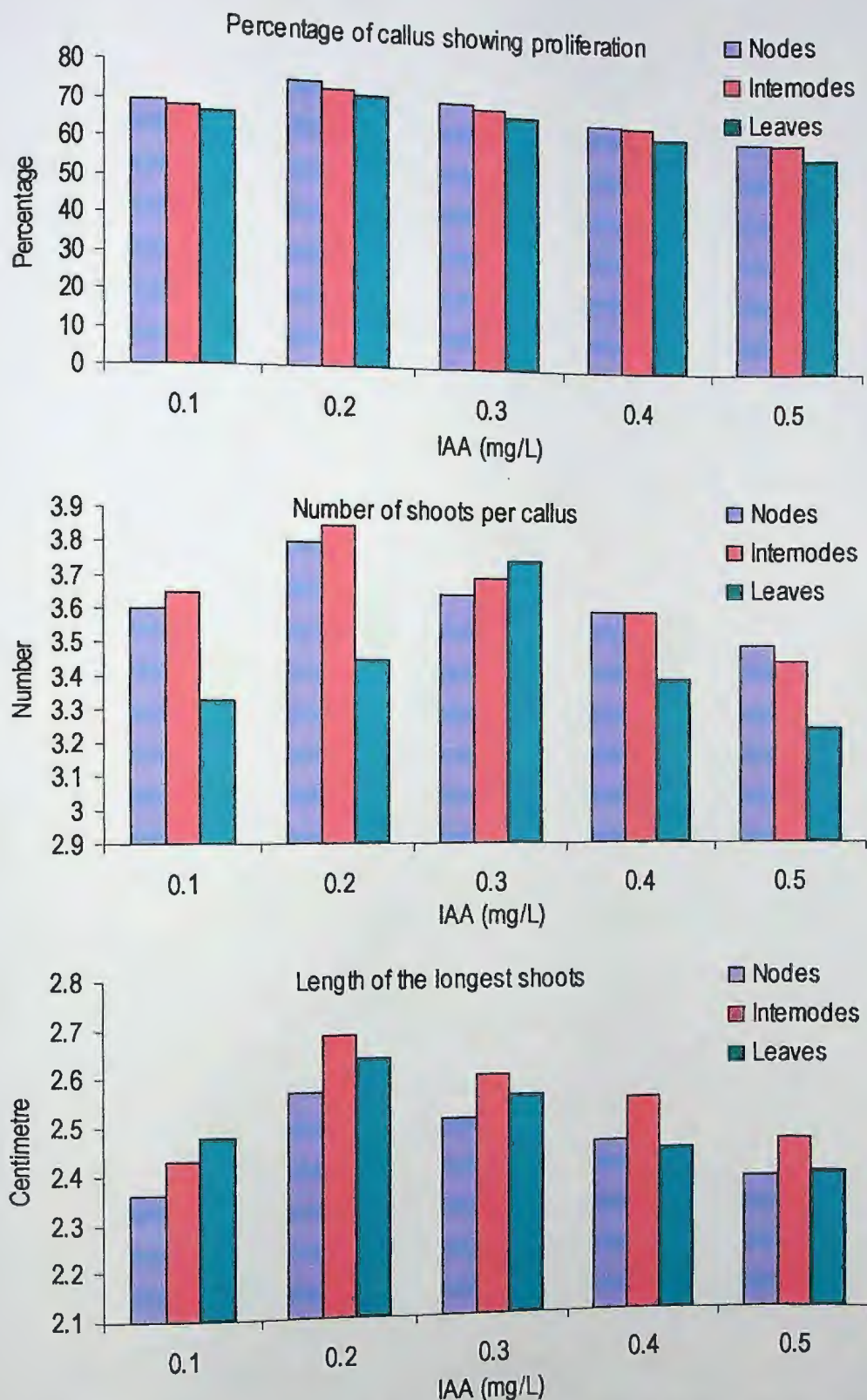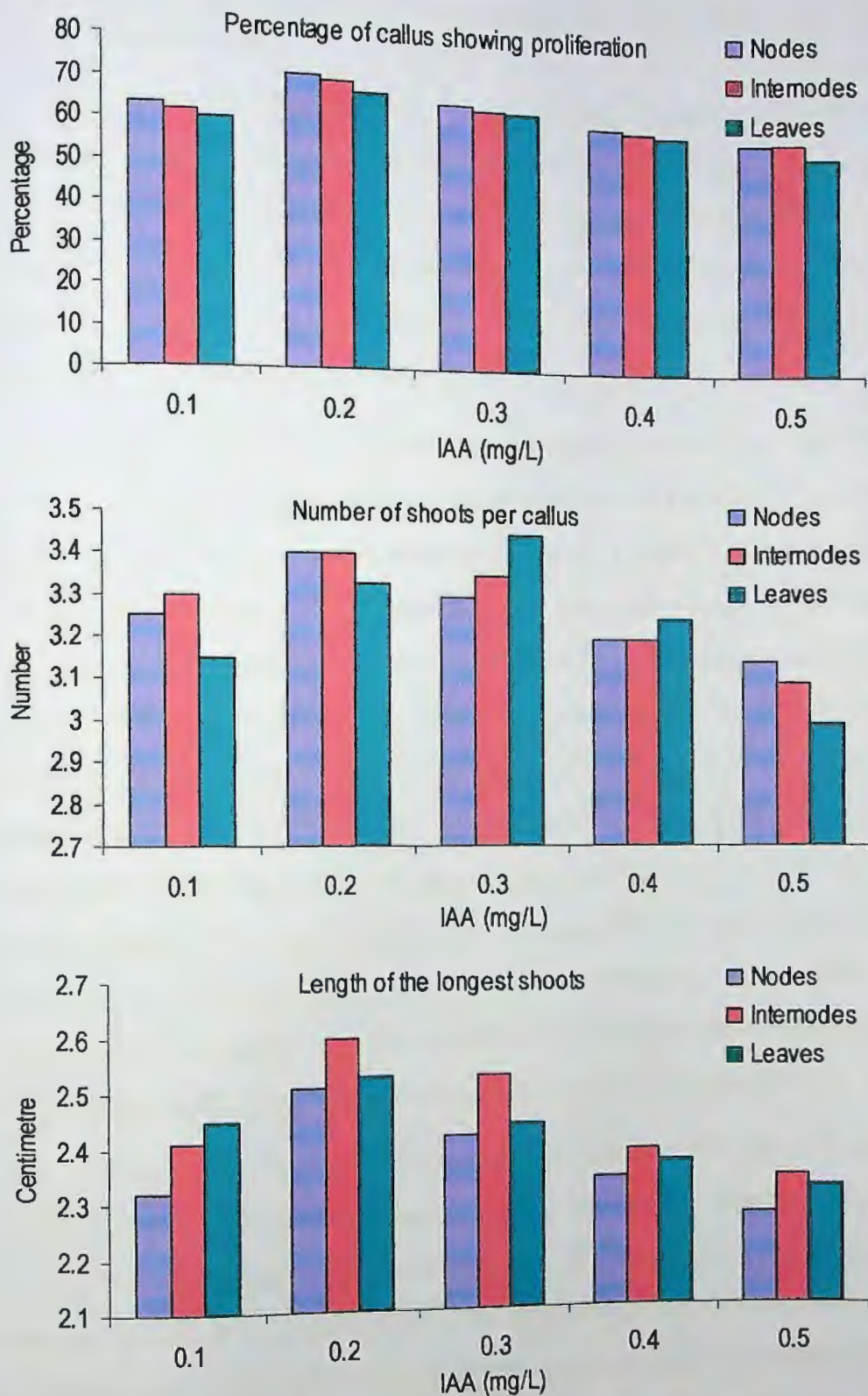ations showed average performances of shoot proliferation of the callus. The effects of BAP (2.0 mg/l) with different concentration of IBA on indirect shoot proliferation from callus of the different explants are presented by histogram in Fig. 32.

BAP (2.5 mg/l) with five different concentration of IBA: Highest percentage of proliferated shoots was found from the callus of nodes which was 73.68 ± 0.79 when BAP (2.5 mg/l) + IBA (0.2 mg/l) was used in the culture media and average number of shoots per callus was 3.50 ± 0.46 and average length of the longest shoots was 2.53 ± 0.52 cm. In same combination of the growth regulators highest percentage of proliferated shoots from the callus of internodes was 68.27 ± 1.22, average number of shoots per callus was 3.50 ±

0.11 and average length of the longest shoots was 2.62 ± 0.23 cm. In case of callus from leaves, highest percentage of proliferated shoots was 65.33 ± 0.94 in same combination of the growth regulators and average number of shoots per callus was 3.40 ± 0.39 and average length of the longest shoots was 2.50 ± 0.34 cm. Other combinations showed average performances of shoot proliferation of the callus. The effects of BAP (2.5 mg/l) with different concentration of IBA on indirect shoot proliferation from callus of the different explants are presented by histogram in Fig. 33.

Table 12. Effects of different concentration of BAP with different concentration of IBA on indirect shoot proliferation from callus of the different explants.

Treatment (mg/l)		Percentage of callus showing proliferation			Average number of shoots per callus			Average length of the longest shoots (cm)		
BAP	IBA	*N	IN	L	N	IN	L	N	IN	L
1.0	0.1	58.70±0.77	55.28±0.48	51.84±1.16	3.35±0.24	3.15±0.67	3.15±0.08	2.32±0.53	2.44±0.17	2.28±0.41
	0.2	63.45±0.46	59.46±1.02	56.55±0.59	3.55±0.17	3.35±0.23	3.25±0.15	2.46±0.24	2.58±0.35	2.42±0.19
	0.3	69.24±0.81	65.17±1.19	62.33±0.58	3.40±0.07	3.20±0.48	3.45±0.12	2.57±0.40	2.69±0.27	2.49±0.36
	0.4	63.85±1.27	59.87±0.56	57.28±0.62	3.35±0.29	3.10±0.16	3.30±0.37	2.44±0.73	2.53±0.09	2.34±0.80
	0.5	57.33±0.92	53.55±0.74	49.80±1.37	3.25±0.13	3.00±0.22	3.10±0.54	2.30±0.45	2.42±0.31	2.26±0.11
1.5	0.1	62.17±0.85	61.45±1.18	60.55±0.64	3.40±0.22	3.25±0.39	3.20±0.55	2.36±0.23	2.45±0.49	2.37±0.51
	0.2	70.24±1.39	68.33±0.13	65.43±0.37	3.55±0.41	3.45±0.50	3.40±0.18	2.51±0.33	2.65±0.28	2.54±0.72
	0.3	69.75±1.04	65.26±0.37	67.60±0.58	3.45±0.13	3.35±0.44	3.55±0.24	2.62±0.17	2.74±0.44	2.62±0.17
	0.4	64.58±1.23	60.37±1.05	62.28±0.48	3.35±0.04	3.25±0.35	3.33±0.72	2.48±0.20	2.61±0.35	2.46±0.28
	0.5	60.45±0.69	57.64±0.32	58.64±1.23	3.30±0.58	3.15±0.20	3.15±0.27	2.35±0.41	2.43±0.32	2.33±0.44
2.0	0.1	71.25±0.97	68.54±0.94	65.54±0.75	3.70±0.28	3.55±0.91	3.33±0.33	2.42±0.04	2.53±0.18	2.40±0.37
	0.2	78.33±0.24	75.43±0.80	71.43±0.53	4.05±0.31	3.75±0.26	3.55±0.18	2.61±0.06	2.72±0.49	2.60±0.22
	0.3	72.84±0.62	69.88±0.28	68.25±1.36	3.80±0.70	3.65±0.52	3.70±0.31	2.68±0.58	2.81±0.21	2.68±0.71
	0.4	71.17±0.90	66.17±0.73	65.47±0.47	3.75±0.35	3.50±0.66	3.40±0.28	2.52±0.33	2.63±0.70	2.51±0.54
	0.5	69.46±1.76	62.35±1.24	61.56±0.22	3.65±0.12	3.40±0.31	3.25±0.55	2.43±0.17	2.52±0.35	2.39±0.23
2.5	0.1	69.55±1.38	63.75±0.50	61.57±0.78	3.35±0.33	3.30±0.57	3.25±0.36	2.35±0.81	2.46±0.28	2.34±0.12
	0.2	73.68±0.79	68.27±1.22	65.33±0.94	3.50±0.46	3.50±0.11	3.40±0.39	2.53±0.52	2.62±0.23	2.50±0.34
	0.3	71.95±1.50	65.42±0.34	62.82±0.48	3.40±0.09	3.35±0.60	3.60±0.48	2.58±0.31	2.70±0.09	2.61±0.28
	0.4	63.70±0.57	61.48±1.09	59.78±0.71	3.25±0.31	3.25±0.48	3.33±0.32	2.44±0.27	2.52±0.13	2.43±0.75
	0.5	61.24±0.28	58.23±1.37	56.42±1.42	3.20±0.27	3.15±0.09	3.20±0.18	2.31±0.49	2.43±0.75	2.33±0.11

*N=Nodes; IN= Internodes; L=Leaves

Fig. 30. Effects of BAP (1.0 mg/l) with different concentration of IBA on indirect shoot proliferation from callus of the different explants of *Stevia rebaudiana*; percentage proliferation (upper), number of shoots per callus (middle), length of longest shoot (lower).
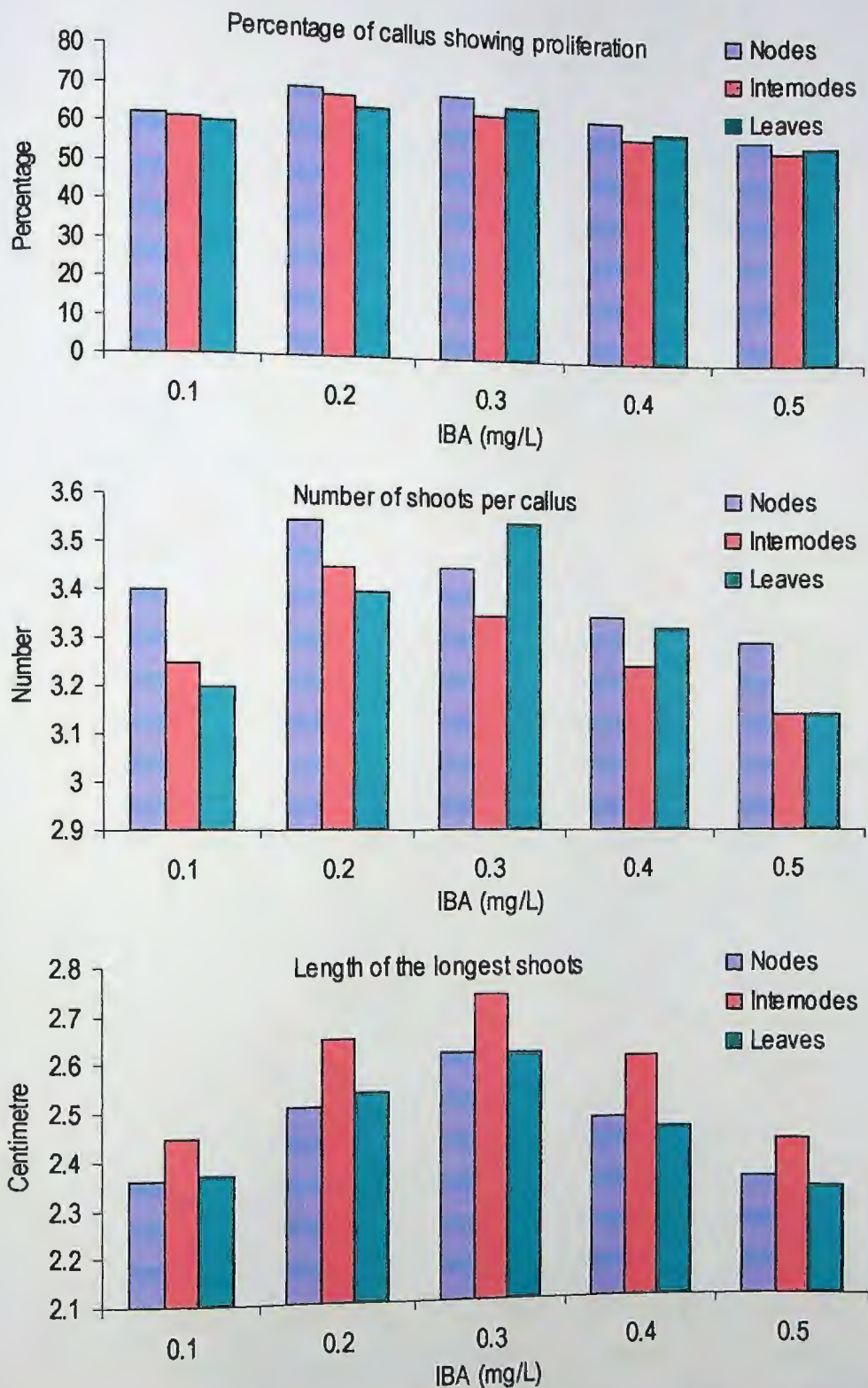
Fig. 31. Effects of BAP (1.5 mg/l) with different concentration of IBA on indirect shoot proliferation from callus of the different explants of *Stevia rebaudiana*; percentage proliferation (upper), number of shoots per callus (middle), length of longest shoot (lower).

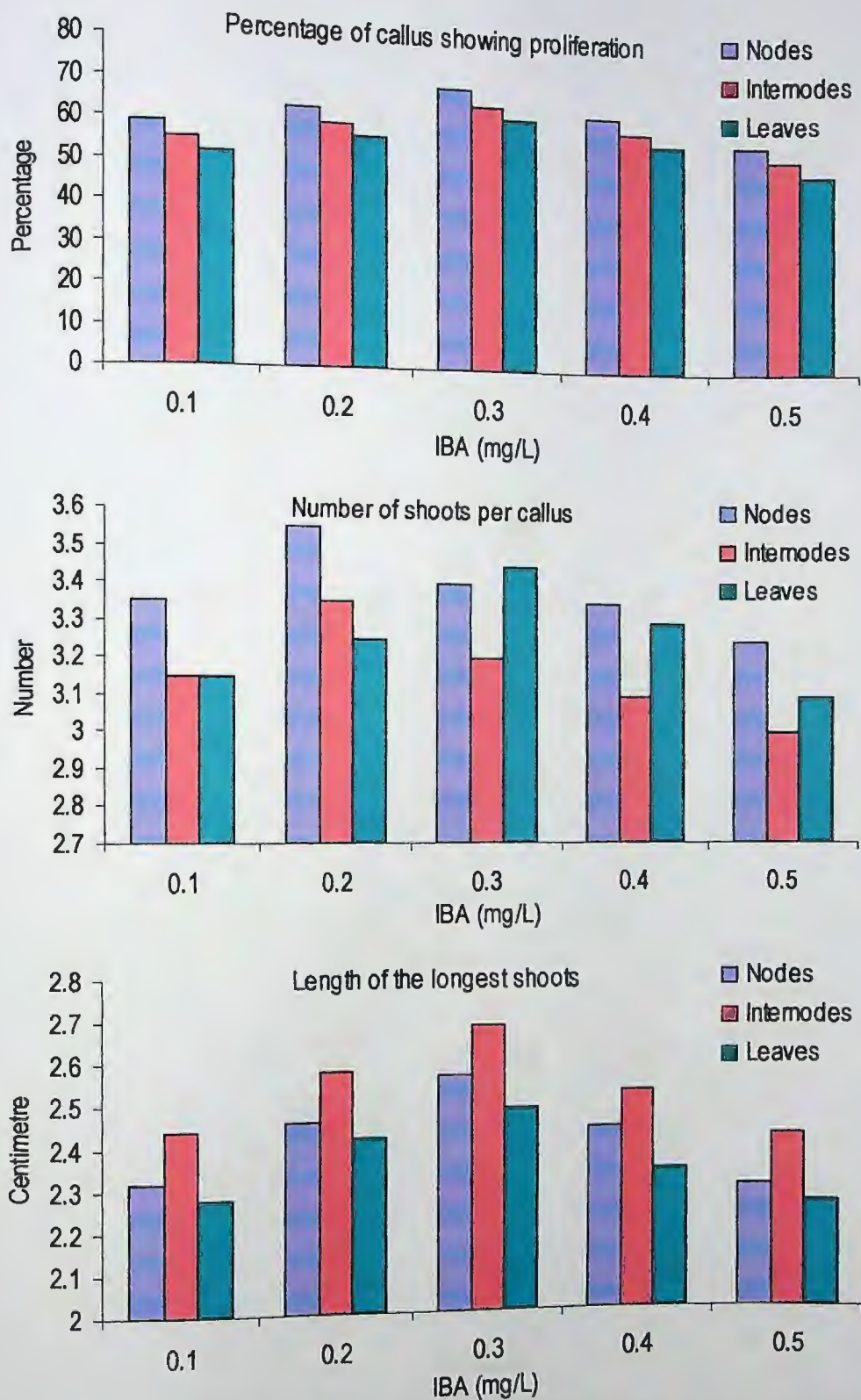**Fig. 32.** Effects of BAP (2.0 mg/l) with different concentration of IBA on indirect shoot proliferation from callus of the different explants of *Stevia rebaudiana*; percentage proliferation (upper), number of shoots per callus (middle), length of longest shoot (lower).

Fig. 33. Effects of BAP (2.5 mg/l) with different concentration of IBA on indirect shoot proliferation from callus of the different explants of *Stevia rebaudiana*; percentage proliferation (upper), number of shoots per callus (middle), length of longest shoot (lower).

3.4.2.3 Effects of different concentration of BAP with different concentration of NAA

Four different concentrations of BAP (1.0 mg/l, 1.5 mg/l, 2.0 mg/l, 2.5 mg/l) with five different concentrations of NAA (0.1 mg/l, 0.2 mg/l, 0.3 mg/l, 0.4 mg/l, 0.5 mg/l) were used in the culture media to find out the combined effects of BAP and NAA on indirect shoots proliferation from the callus of nodes, internodes and leaves and the results are presented in Table 13. In most of the cases NAA (0.2 mg/l) or IAA (0.3 mg/l) with different concentration of BAP showed the best result.

BAP (1.0 mg/l) with five different concentration of NAA: In case of callus from nodes the highest percentage of proliferated shoots was observed when BAP (1.0 mg/l) + NAA (0.2 mg/l) was used in the culture media which was 78.64 ± 0.69. In this combination average number of shoots per callus was 3.60 ± 0.13 and average length of the longest shoots was 2.40 ± 0.06 cm. But in case of callus of internodes, the highest percentage of proliferated shoots was 72.46 ± 1.40 in same combination of the growth regulators and average number of shoots per callus was 3.50 ± 0.29 and average length of the longest shoots was 2.47 ± 0.33 cm. Again in the combination of BAP (1.0 mg/l) + NAA (0.3 mg/l) in the culture media, the highest percentage of proliferated shoots from the callus of leaves was 67.33 ± 0.56, average number of shoots per callus was 3.55±0.17 and average length of the longest shoots was 2.53 ± 0.28 cm. Other combinations showed average performances of shoot proliferation of the callus. The effects of BAP (1.0 mg/l) with different concentration of NAA on indirect shoot proliferation from callus of the different explants are presented by histogram in Fig. 34.

BAP (1.5 mg/l) with five different concentration of NAA: When BAP (1.5 mg/l) + NAA (0.2 mg/l) was used in the culture media, highest percentage of proliferated shoots was recorded from the callus of nodes which was 86.22 ± 1.24, average number of shoots per callus was 3.70 ± 0.29 and average length of the longest shoots was 2.45 ± 0.37 cm. Highest percentage of proliferated shoots from the callus of internodes was 81.26 ± 0.17 in

the same combination of BAP with NAA and average number of shoots per callus was 3.67 ± 0.34 and average length of the longest shoots was 2.56 ± 0.22 cm. Again in the combination of BAP (1.0 mg/l) + NAA (0.3 mg/l) in the culture media, the highest percentage of proliferated shoots from the callus of leaves was 77.58 ± 0.94, average number of shoots per callus was 3.75 ± 0.05 and average length of the longest shoots was 2.64 ± 0.43 cm. Other combinations showed average performances of shoot proliferation of the callus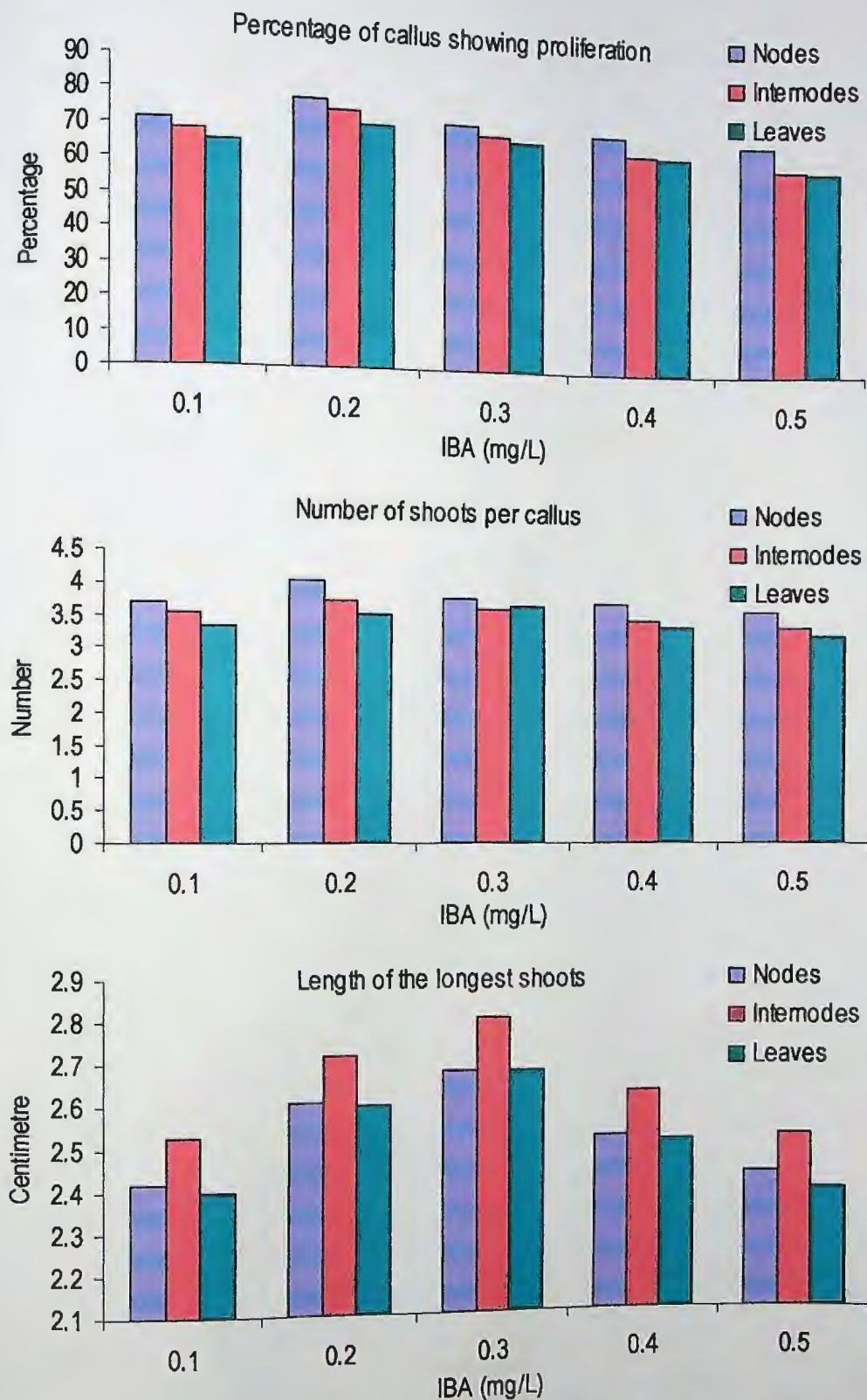. The effects of BAP (1.5 mg/l) with different concentration of NAA on indirect shoot proliferation from callus of the different explants are presented by histogram in Fig. 35.

BAP (2.0 mg/l) with five different concentration of NAA: BAP (2.0 mg/l) + NAA (0.2 mg/l) in the culture media showed the highest percentage of proliferated shoots from the callus of nodes which was 94.70 ± 0.72. This is the best result among the BAP + NAA combinations. In this combination average number of shoots per callus was 4.25 ± 0.30 and average length of the longest shoots was 2.58 ± 0.12 cm. In the same combination of the growth regulators, highest percentage of proliferated shoots from the callus of internodes was 89.15 ± 1.05, average number of shoots per callus was 4.20 ± 0.16 and average length of the longest shoots was 2.73 ± 0.24 cm. But in case of callus from leaves, the highest percentage of proliferated shoots was observed in the same combination of BAP and NAA in the culture media which was 84.73 ± 1.17. In this combination average number of shoots per callus was 3.67 ± 0.42 and average length of the longest shoots was 2.57 ± 0.36 cm. Other combinations showed average performances of shoot proliferation of the callus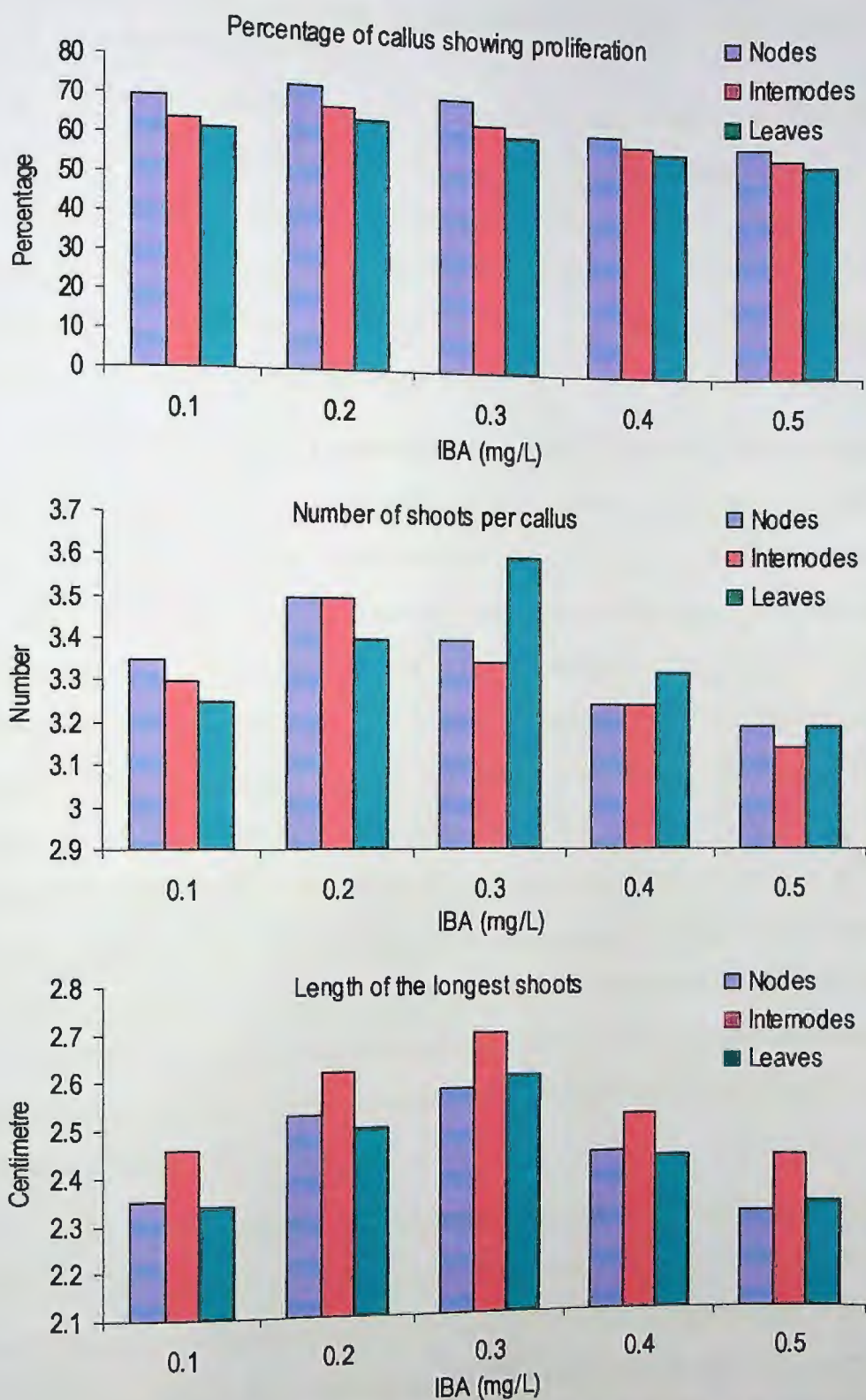. The effects of BAP (2.0 mg/l) with different concentration of NAA on indirect shoot proliferation from callus of the different explants are presented by histogram in Fig. 36.

BAP (2.5 mg/l) with five different concentration of NAA: Highest percentage of proliferated shoots was found from the callus of nodes which was 84.67 ± 1.28 when BAP (2.5 mg/l) + NAA (0.2 mg/l) was used in the culture media and average number of shoots per callus was 3.65 ± 0.44 and average length of the longest shoots was 2.45 ± 0.08 cm. In same combination of the growth regulators highest percentage of proliferated shoots from

the callus of internodes was 79.84 ± 0.82, average number of shoots per callus was 3.45 ± 0.18 and average length of the longest shoots was 2.62 ± 0.07 cm. In case of callus from leaves, highest percentage of proliferated shoots was 78.45 ± 0.65 in same combination of the growth regulators and average number of shoots per callus was 3.55 ± 0.26 and average length of the longest shoots was 2.50 ± 0.39 cm. Other combinations showed average performances of shoot proliferation of the callus. The effects of BAP (2.5 mg/l) with different concentration of NAA on indirect shoot proliferation from callus of the different explants are presented by histogram in Fig. 37.

Table 13. Effects of different concentration of BAP with different concentration of NAA on indirect shoot proliferation from callus of the different explants of *Stevia rebaudiana*.

Treatment (mg/l)		Percentage of callus showing proliferation			Average number of shoots per callus			Average length of the longest shoots (cm)		
BAP	NAA	*N	IN	L	N	IN	L	N	IN	L
1.0	0.1	70.26±1.03	65.38±0.95	56.25±0.48	3.45±0.28	3.33±0.44	3.20±0.08	2.26±0.12	2.36±0.08	2.30±0.37
	0.2	78.64±0.69	72.46±1.40	63.84±1.32	3.60±0.13	3.50±0.29	3.33±0.22	2.40±0.06	2.47±0.33	2.44±0.15
	0.3	74.86±1.31	69.17±1.09	67.33±0.56	3.50±0.31	3.40±0.27	3.55±0.17	2.52±0.28	2.51±0.21	2.53±0.28
	0.4	67.17±0.80	63.63±0.54	62.20±0.88	3.40±0.58	3.25±0.19	3.30±0.34	2.38±0.33	2.42±0.17	2.36±0.38
	0.5	62.38±0.59	58.82±0.48	55.42±1.24	3.35±0.06	3.15±0.52	3.15±0.31	2.24±0.13	2.37±0.28	2.25±0.43
1.5	0.1	74.46±0.66	70.58±0.89	65.86±1.33	3.50±0.23	3.40±0.18	3.33±0.45	2.32±0.28	2.41±0.44	2.33±0.19
	0.2	86.22±1.24	81.26±0.17	76.38±0.59	3.70±0.29	3.67±0.34	3.60±0.11	2.45±0.37	2.56±0.22	2.47±0.56
	0.3	81.39±1.10	76.35±0.68	77.58±0.94	3.60±0.18	3.50±0.09	3.75±0.05	2.58±0.22	2.49±0.14	2.64±0.43
	0.4	76.45±0.48	71.42±1.22	68.26±0.34	3.50±0.79	3.33±0.27	3.67±0.22	2.42±0.03	2.43±0.21	2.46±0.52
	0.5	71.63±0.92	66.25±1.16	62.45±1.08	3.40±0.20	3.25±0.33	3.25±0.85	2.28±0.19	2.38±0.33	2.30±0.25
2.0	0.1	84.25±0.44	78.46±0.95	73.42±1.38	3.80±0.11	3.80±0.25	3.45±0.45	2.38±0.33	2.46±0.19	2.37±0.77
	0.2	94.70±0.72	89.15±1.05	84.73±1.17	4.25±0.30	4.20±0.16	3.67±0.42	2.58±0.12	2.73±0.24	2.57±0.36
	0.3	89.67±1.43	84.26±0.86	76.26±0.49	4.00±0.46	3.90±0.59	3.90±0.18	2.63±0.41	2.62±0.32	2.72±0.17
	0.4	85.15±0.59	78.82±0.74	70.67±0.81	3.85±0.08	3.75±0.26	3.70±0.37	2.45±0.09	2.54±0.18	2.49±0.22
	0.5	79.22±0.75	72.52±1.38	67.15±0.62	3.65±0.63	3.50±0.14	3.33±0.54	2.36±0.15	2.42±0.49	2.37±0.36
2.5	0.1	78.43±0.86	71.78±0.59	69.25±1.47	3.45±0.29	3.33±0.05	3.33±0.33	2.30±0.27	2.43±0.34	2.35±0.57
	0.2	84.67±1.28	79.84±0.82	78.45±0.65	3.65±0.44	3.45±0.18	3.55±0.26	2.45±0.08	2.62±0.07	2.50±0.39
	0.3	80.33±0.48	74.33±0.77	71.33±1.28	3.50±0.38	3.40±0.44	3.75±0.07	2.49±0.19	2.56±0.11	2.64±0.24
	0.4	74.88±1.09	69.45±1.45	66.58±0.61	3.45±0.20	3.25±0.37	3.45±0.66	2.36±0.41	2.45±0.59	2.43±0.18
	0.5	66.45±1.14	64.57±0.31	61.20±0.58	3.30±0.18	3.10±0.51	3.15±0.43	2.26±0.20	2.38±0.34	2.32±0.07

*N=Nodes; IN= Internodes; L=Leaves

Fig. 34. Effects of BAP (1.0 mg/l) with different concentration of NAA on indirect shoot proliferation from callus of the different explants of *Stevia rebaudiana*; percentage proliferation (upper), number of shoots per callus (middle), length of longest shoot (lower).

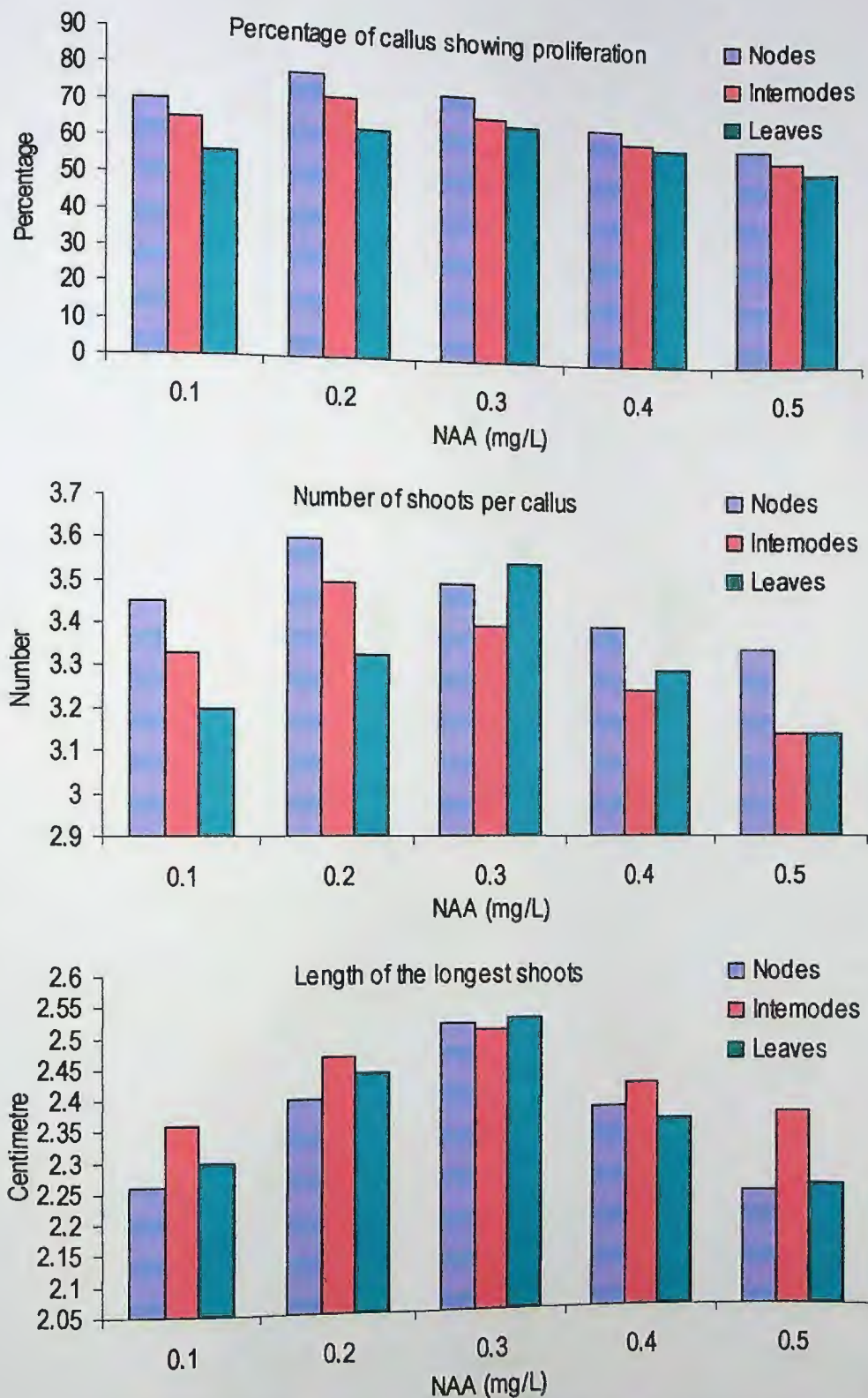**Fig.35.** Effects of BAP (1.5 mg/l) with different concentration of NAA on indirect shoot proliferation from callus of the different explants of *Stevia rebaudiana*; percentage proliferation (upper), number of shoots per callus (middle), length of longest shoot (lower).
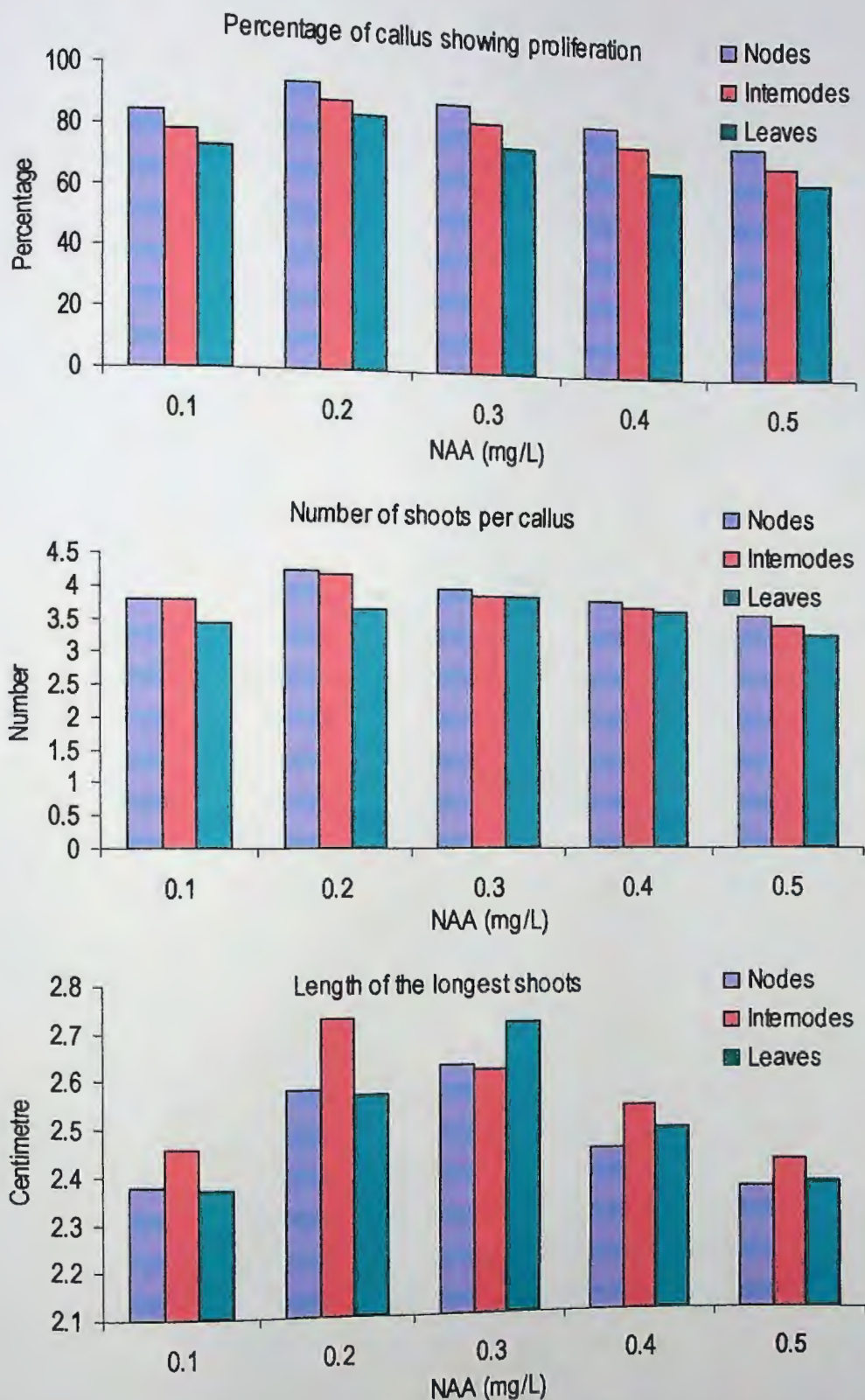
Fig. 36. Effects of BAP (2.0 mg/l) with different concentration of NAA on indirect shoot proliferation from callus of the different explants of *Stevia rebaudiana*; percentage proliferation (upper), number of shoots per callus (middle), length of longest shoot (lower)..

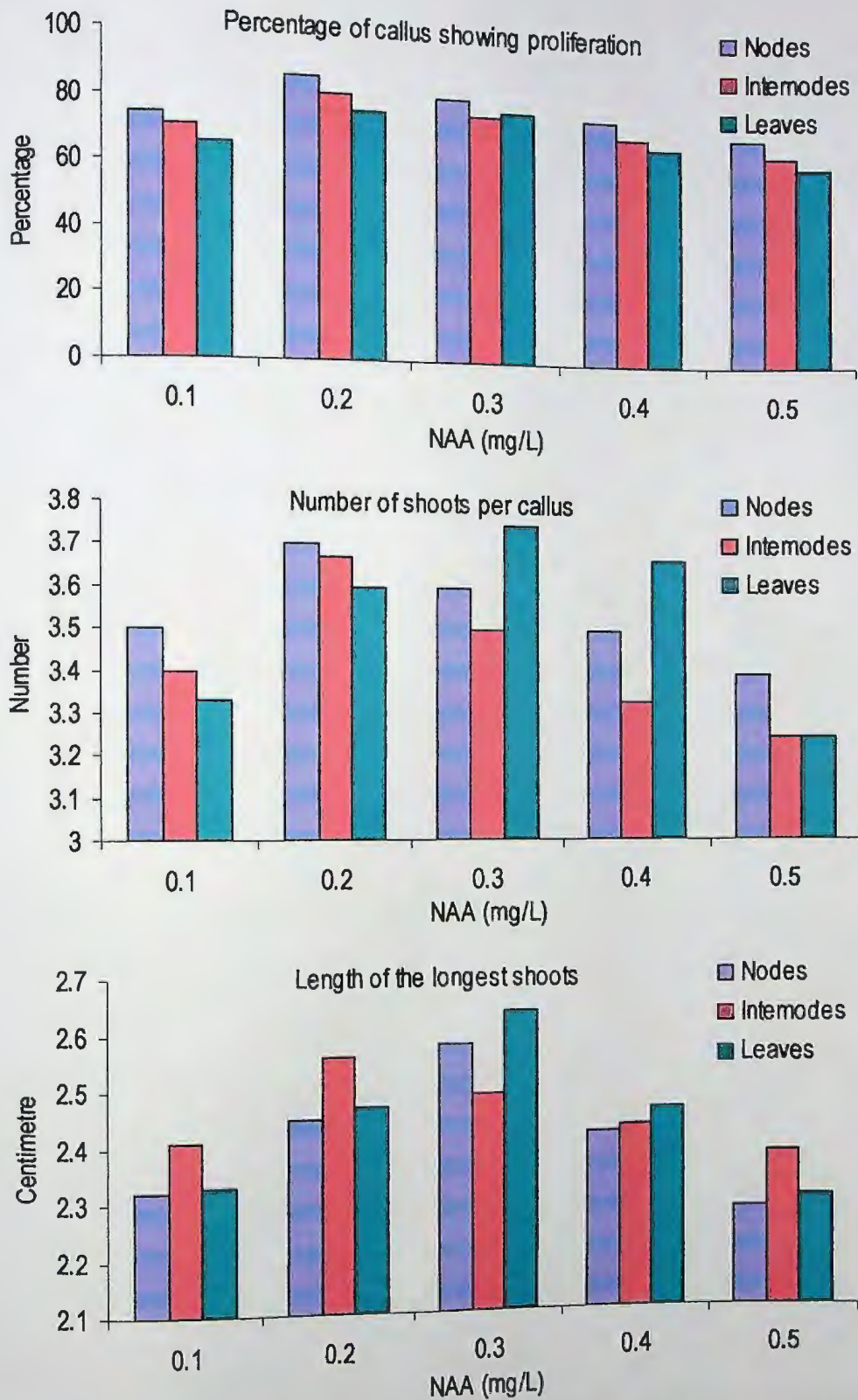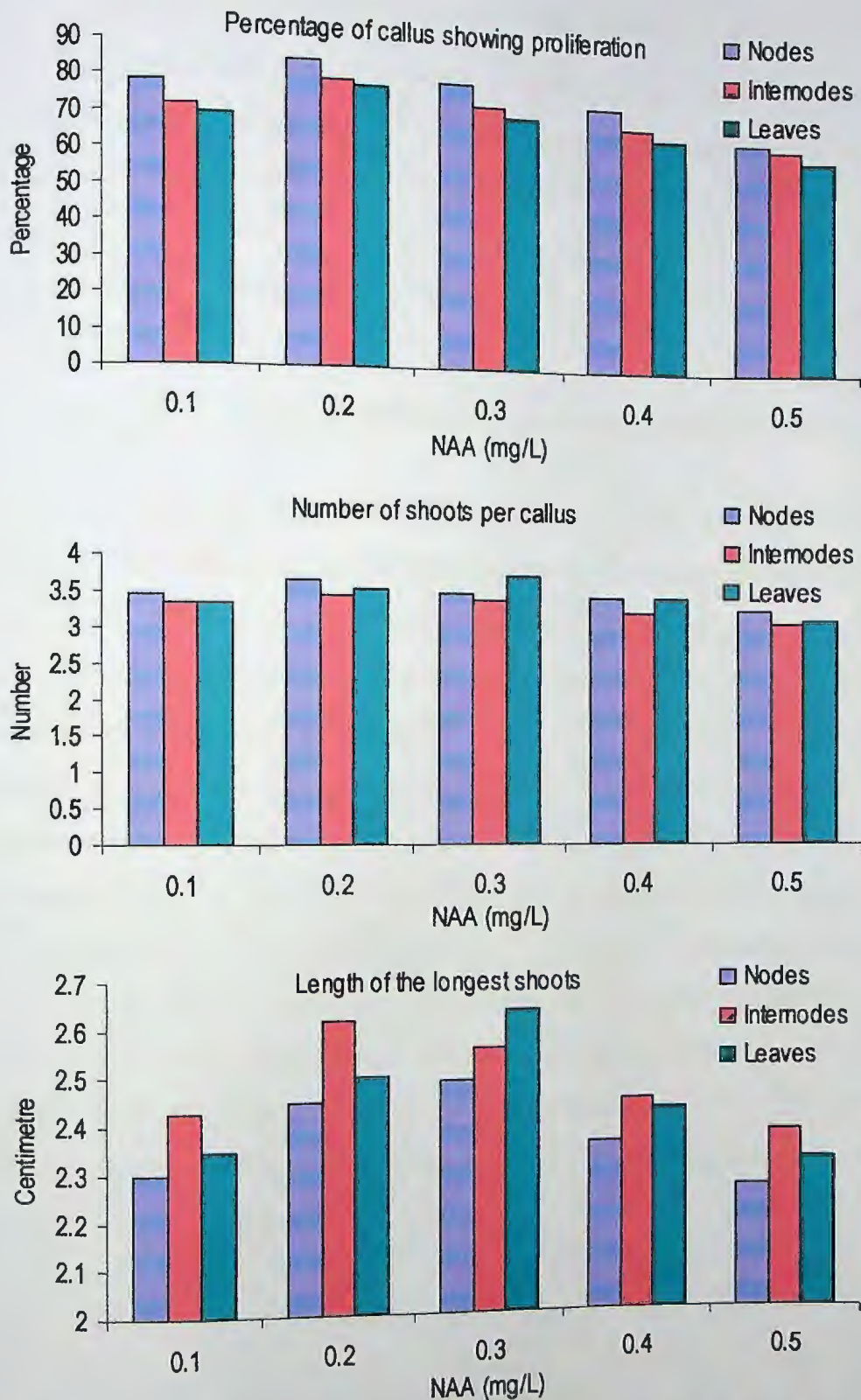**Fig. 37.** Effects of BAP (2.5 mg/l) with different concentration of NAA on indirect shoot proliferation from callus of the different explants of *Stevia rebaudiana*; percentage proliferation (upper), number of shoots per callus (middle), length of longest shoot (lower).

3.5 Root proliferation from induced shoots of the different explants

Three types of Auxins (IAA, IBA, NAA), each with different concentration were used in the culture media for the proliferation of roots from new induced shoots of the different explants and the results are presented in Table 14. In all cases NAA was found the most effective than IAA and IBA on root proliferation and induced shoots from nodes showed the highest percentage of proliferated roots compared to induced shoots from internodes and leaves (Plate 4).

3.5.1 Effects of IAA on root proliferation from induced shoots of the different explants

Six different concentrations of IAA (0.1 mg/l, 0.2 mg/l, 0.3 mg/l, 0.4 mg/l, 0.5 mg/l, 1.0 mg/l) were used in the culture media for root proliferation of the induced shoots. Highest percentage of proliferated roots was recorded 87.75 ± 0.54 from induced shoots of nodes with IAA (0.3 mg/l) in the culture media. Average number of roots per shoot was 6.40 ± 0.32 and average length of the longest root was 2.33 ± 0.07 cm. In this concentration, highest percentage of proliferated roots was recorded 86.38 ± 1.15 from induced shoots of internodes and average number of roots per shoot was 5.51 ± 0.24 and average length of the longest root was 2.31 ± 0.40 cm. In case of leaves, highest percentage of proliferated roots from induced shoots was recorded 84.92 ± 0.47 in same concentration of IAA, average number of roots per shoot was 5.36 ± 0.69 and average length of the longest root was 2.28 ± 0.11 cm. Other concentrations of IAA also showed better performances of root proliferation of the new induced shoots. The effects of different concentration of IAA on root proliferation from induced shoots of the different explants are presented by histogram graph in Fig. 38.

3.5.2 Effects of IBA on root proliferation from induced shoots of the different explants

Same as IAA, six different concentrations of IBA (0.1 mg/l, 0.2 mg/l, 0.3 mg/l, 0.4 mg/l, 0.5 mg/l, 1.0 mg/l) were used in the culture media for root proliferation of the induced shoots. When IBA (0.3 mg/l) was used in the culture, highest percentage of proliferated roots from the induced shoots of nodes was recorded 90.17 ± 0.88, average number of roots per shoot was 7.20 ± 0.32 and average length of the longest root was 2.26 ± 0.17 cm. In this concentration of IBA, highest percentage of proliferated roots from shoots of internodes was 90.73 ± 0.28, average number of roots per shoot was 8.15 ± 0.19 and average length of the longest root was 2.29 ± 0.22 cm. Again in same concentrations of IBA, highest percentage of proliferated roots from the induced shoots of leaves was recorded 88.29 ± 0.75, average number of roots per shoot was 8.77 ± 0.41 and average length of the longest root was 2.32 ± 0.30 cm. Other concentrations of IBA also showed better performances of root proliferation of the new induced shoots. The effects of different concentration of IAA on root proliferation from induced shoots of the different explants are presented by histogram in Fig. 39.

3.5.3 Effects of NAA on root proliferation from induced shoots of the different explants

Again six different concentrations of NAA (0.1 mg/l, 0.2 mg/l, 0.3 mg/l, 0.4 mg/l, 0.5 mg/l, 1.0 mg/l) were used in the culture media for root proliferation of the induced shoots. NAA was found most effective on root proliferation and percentage of root proliferation from induced shoots of different explants was also high compared to other two Auxins (IAA, IBA). Highest percentage of proliferated roots was recorded when NAA (0.2 mg/l) was used in the culture media which was 93.33 ± 0.48 from induced shoots of nodes. This is the best result of root proliferation from induced shoots among different Auxins used in the culture media. Average number of roots per shoot was also high which was 10.40±0.62 and average length of the longest root was 2.30 ± 0.15 cm. Highest percentage of proliferated roots from induced shoots of internodes was recorded 92.45 ± 0.72 in same concentration of NAA in

the culture media and average number of roots per shoot was 9.08 ± 0.28 and average length of the longest root was 2.38 ± 0.08 cm. Again in the same concentration of NAA, highest percentage of proliferated roots from induced shoots of leaves was recorded 92.21 ± 0.33, average number of roots per shoot was also high which was 10.23 ± 0.55 and average length of the longest root was 2.36 ± 0.13 cm. Other concentrations of NAA also showed better performances of root proliferation of the new induced shoots. The effects of different concentration of NAA on root proliferation from induced shoots of the different explants are presented by histogram in Fig. 40.

Table 14. Effects of different concentration of IAA, IBA and NAA on root proliferation from induced shoots of the different explants of *Stevia rebaudiana*.

Treatment (mg/l)	Percentage of shoots showing root proliferation			Average number of roots per shoot			Average length of the longest roots (cm)		
	N*	IN	L	N	IN	L	N	IN	L
IAA									
0.1	76.55±0.34	78.16±1.31	74.40±0.86	4.67±0.13	3.88±0.72	3.41±0.35	2.15±0.12	2.18±0.20	2.15±0.31
0.2	86.75±0.26	86.25±0.80	83.87±0.35	7.75±0.44	5.48±0.29	5.22±0.87	2.24±0.32	2.29±0.09	2.15±0.55
0.3	87.75±0.54	86.38±1.15	84.92±0.47	6.40±0.32	5.51±0.24	5.36±0.69	2.33±0.07	2.31±0.40	2.28±0.11
0.4	76.33±0.81	78.29±0.58	79.11±0.18	4.33±0.56	4.81±0.60	4.44±0.59	2.26±0.19	2.25±0.38	2.22±0.16
0.5	68.80±1.22	64.33±0.39	65.72±0.23	3.85±0.71	4.07±0.37	3.92±0.44	2.20±0.34	2.20±0.54	2.24±0.41
1.0	61.25±0.39	62.48±0.27	61.38±0.48	3.25±0.34	3.20±0.92	3.55±0.85	2.10±0.38	2.15±0.19	2.14±0.23
IBA									
0.1	78.67±0.76	74.89±0.44	73.19±0.61	5.80±0.92	4.78±0.18	4.34±0.31	2.10±0.49	2.12±0.08	2.13±0.34
0.2	89.85±0.45	88.21±0.91	85.45±1.57	9.25±0.44	7.50±0.78	5.19±0.25	2.18±0.33	2.38±0.86	2.28±0.18
0.3	90.17±0.88	90.73±0.28	88.29±0.75	7.20±0.32	8.15±0.19	8.77±0.41	2.26±0.17	2.29±0.22	2.32±0.30
0.4	79.42±0.31	75.43±1.39	74.58±0.45	4.85±0.90	5.32±0.36	6.16±0.28	2.20±0.05	2.22±0.31	2.28±0.26
0.5	70.20±1.21	70.55±0.87	64.93±0.23	4.10±0.56	3.45±0.20	4.29±0.93	2.15±0.35	2.15±0.49	2.11±0.54
1.0	64.76±1.04	66.81±1.20	61.22±0.17	3.55±0.88	3.03±0.41	3.45±0.08	2.05±0.28	2.12±0.38	2.12±0.39
NAA									
0.1	84.60±0.61	88.52±0.54	83.65±0.90	6.35±0.37	7.86±0.89	6.22±0.57	2.23±0.30	2.25±0.17	2.25±0.44
0.2	93.33±0.48	92.45±0.72	92.21±0.33	10.40±0.62	9.08±0.28	10.23±0.55	2.30±0.15	2.38±0.08	2.36±0.13
0.3	91.40±0.11	90.27±0.35	91.35±0.48	8.17±0.48	7.49±0.59	8.27±0.18	2.42±0.31	2.40±0.13	2.44±0.34
0.4	80.15±1.25	84.58±0.55	82.43±0.61	5.45±0.59	5.51±0.15	6.09±0.30	2.32±0.08	2.28±0.33	2.25±0.58
0.5	71.55±0.69	68.03±0.21	61.33±0.94	4.70±0.12	4.37±0.66	4.62±0.86	2.25±0.13	2.26±0.58	2.22±0.36
1.0	67.48±0.40	62.10±0.56	63.35±1.27	3.85±0.37	3.57±0.70	3.13±0.45	2.18±0.28	2.15±0.42	2.16±0.18

*N=Nodes; IN= Internodes; L=Leaves
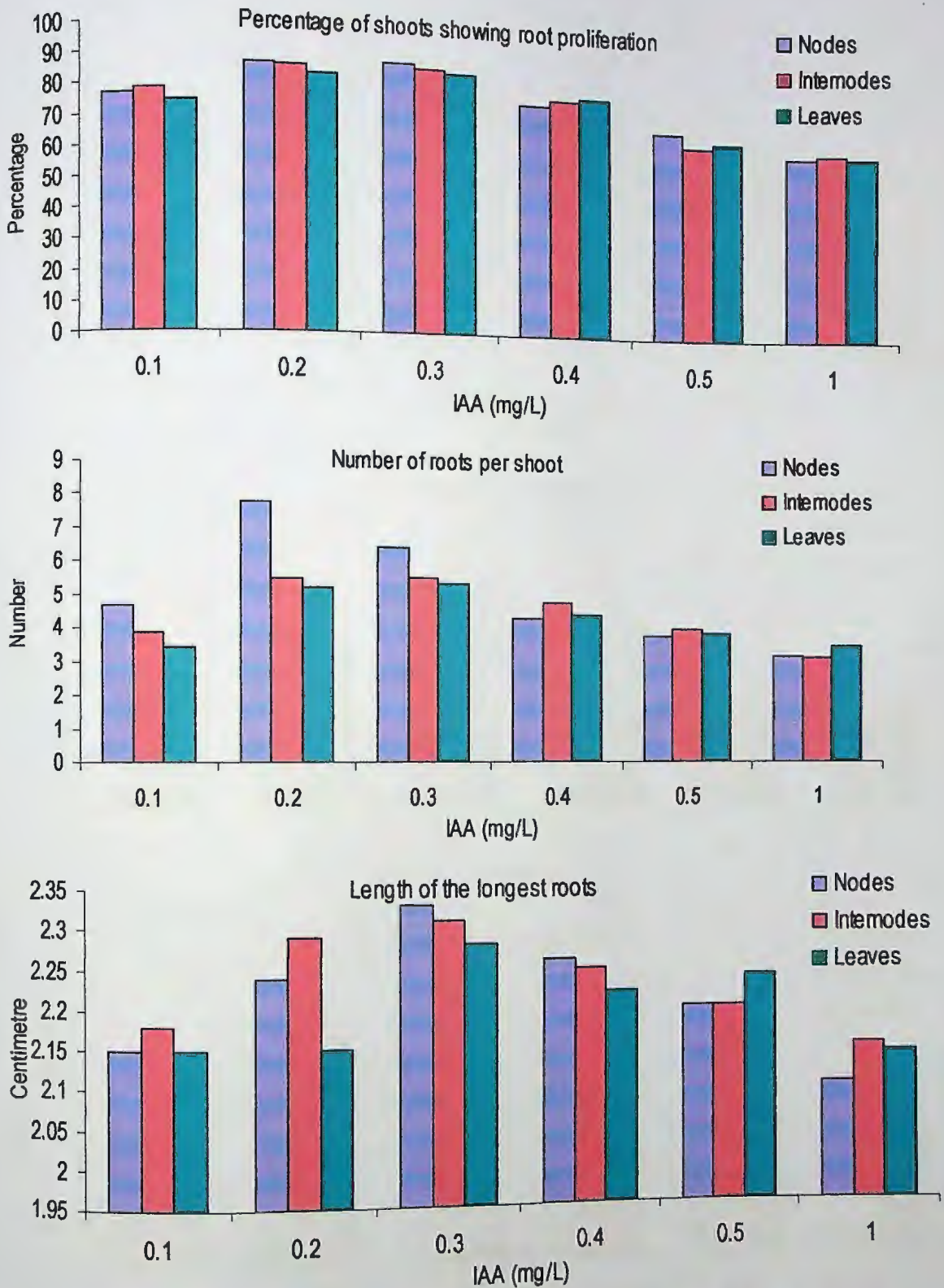
Fig. 38. Effects of different concentration of IAA on root proliferation from induced shoots of the different explants of *Stevia rebaudiana*; percentage root proliferation (upper), number of roots per shoot (middle), length of longest root (lower).

Fig. 39. Effects of different concentration of IBA on root proliferation from induced shoots of the different explants of *Stevia rebaudiana*; percentage root proliferation (upper), number of roots per shoot (middle), length of longest root (lower).

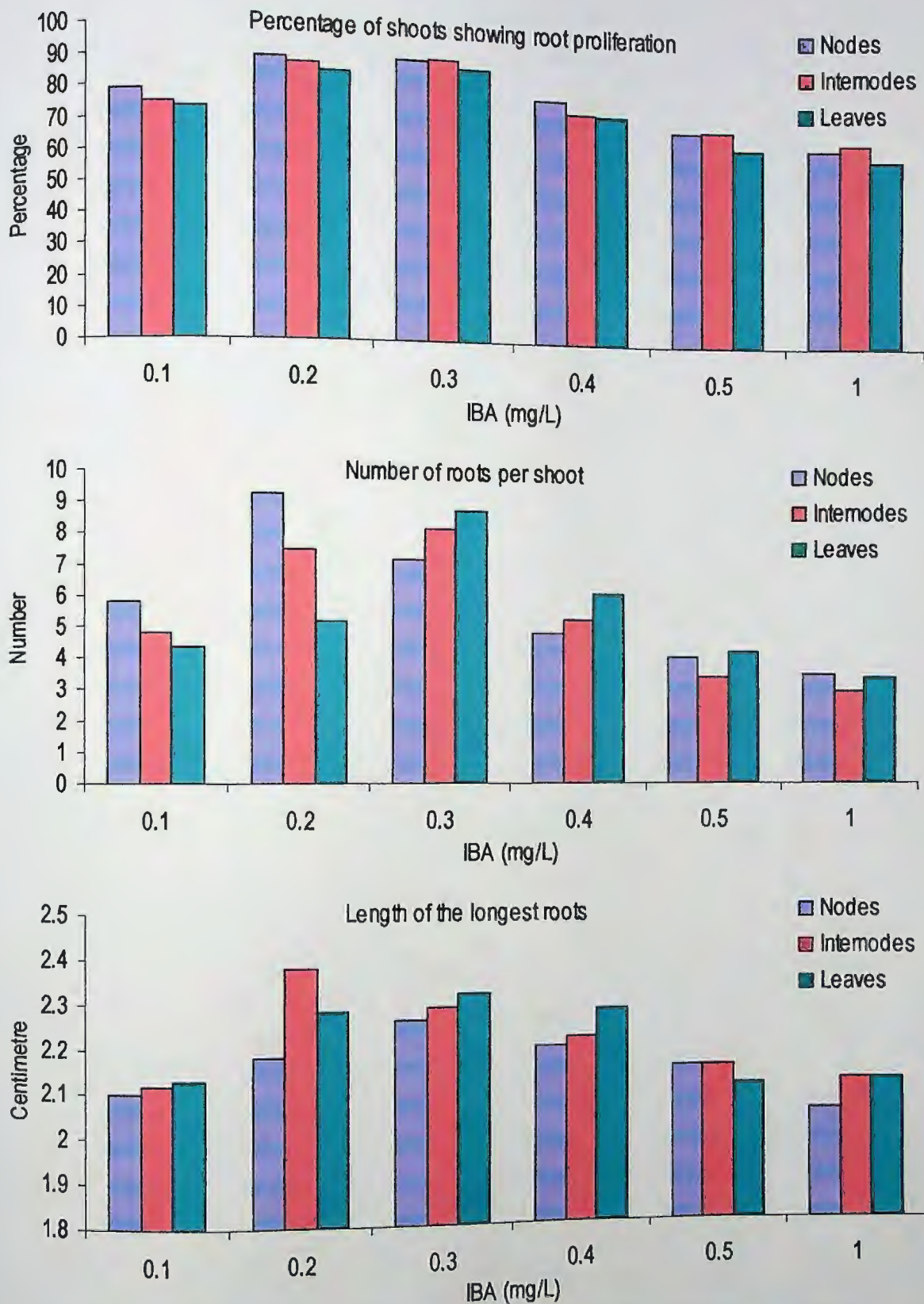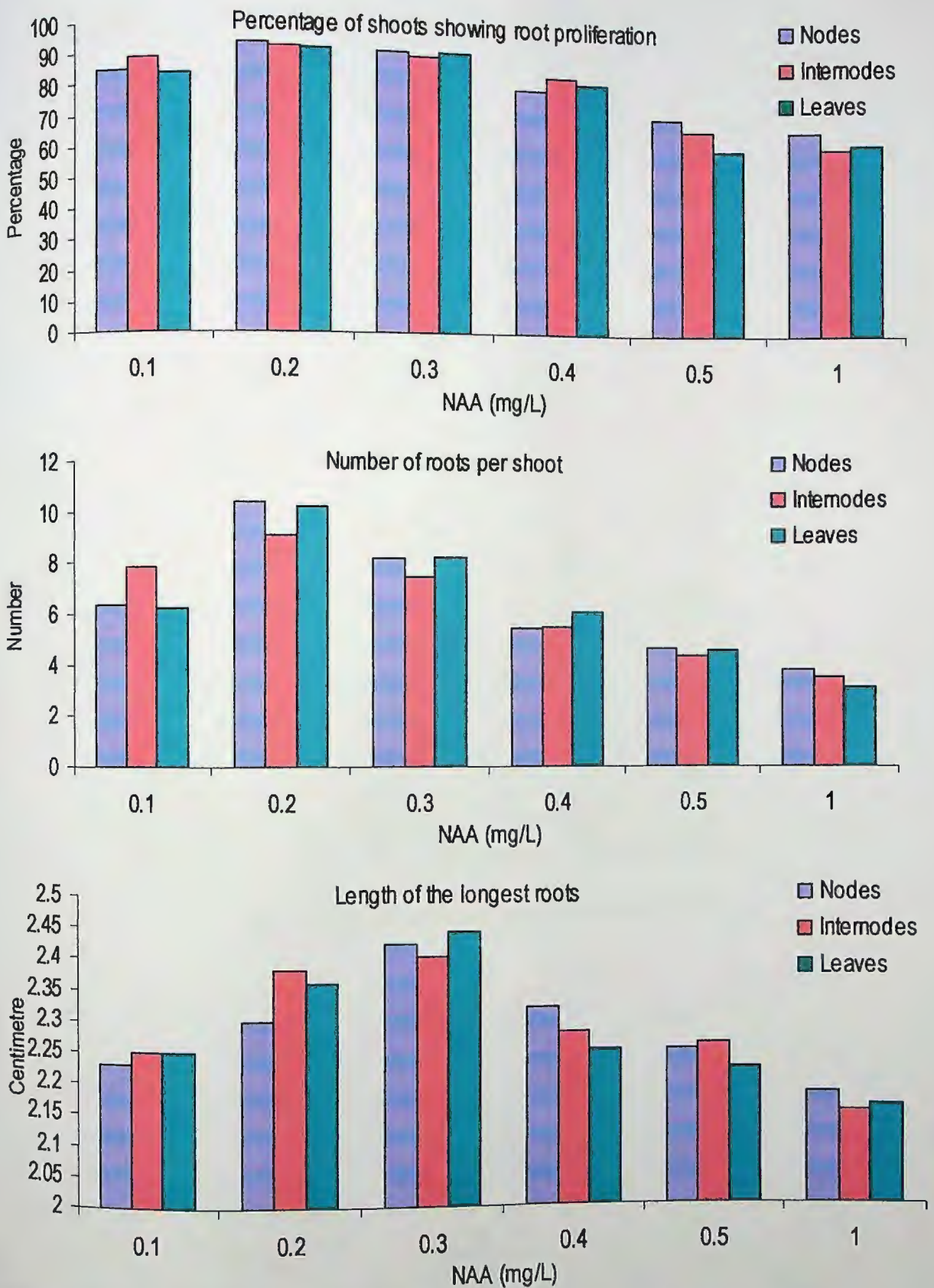**Fig. 40.** Effects of different concentration of NAA on root proliferation from induced shoots of the different explants of *Stevia rebaudiana*; percentage root proliferation (upper), number of roots per shoot (middle), length of longest root (lower).

Plate 4. Root proliferation from induced shoots of *Stevia rebaudiana*

3.6   Establishment of plantlets derived from the different explants

The regenerated new plantlets derived from the different explants (nodes, internodes, leaves) were acclimatized under natural environment and percentages of survived plantlets are presented in Table 15. Plantlets derived from nodes showed highest percentage of survival rate compared to plantlets derived from internodes and leaves under natural environment (Plate 5 and 6).

Under natural environment percentage of survived plantlets derived from nodes was recorded 86.17% after 1st week, 83.56% after 2nd and 82.64% after 3rd week of transplantation and remained 82.64% after 4th week. Percentage of survived plantlets derived from internodes was recorded 84.79%, 82.61% and 81.38% after 1st, 2nd and 3rd week of transplantation respectively and it remained 81.38% after 4th week. Again percentage of survived plantlets derived from nodes was 81.62% after 1st week of transplantation and it became 78.30% and 76.53% after 2nd and 3rd week and remained 76.53% after 4th week. Percentages of survived plantlets derived from the different explants after transplantation under natural environment are presented by histogram graph in Fig. 40.

Table 15. Percentage of survived plantlets derived from the different explants after transplantation under natural environment.

Weeks	Percentage of survived plantlets		
	Nodes	Internodes	Leaves
After 1st week	86.17	84.79	81.62
After 2nd week	83.56	82.61	78.30
After 3rd week	82.64	81.38	76.53
After 4th week	82.64	81.38	76.53
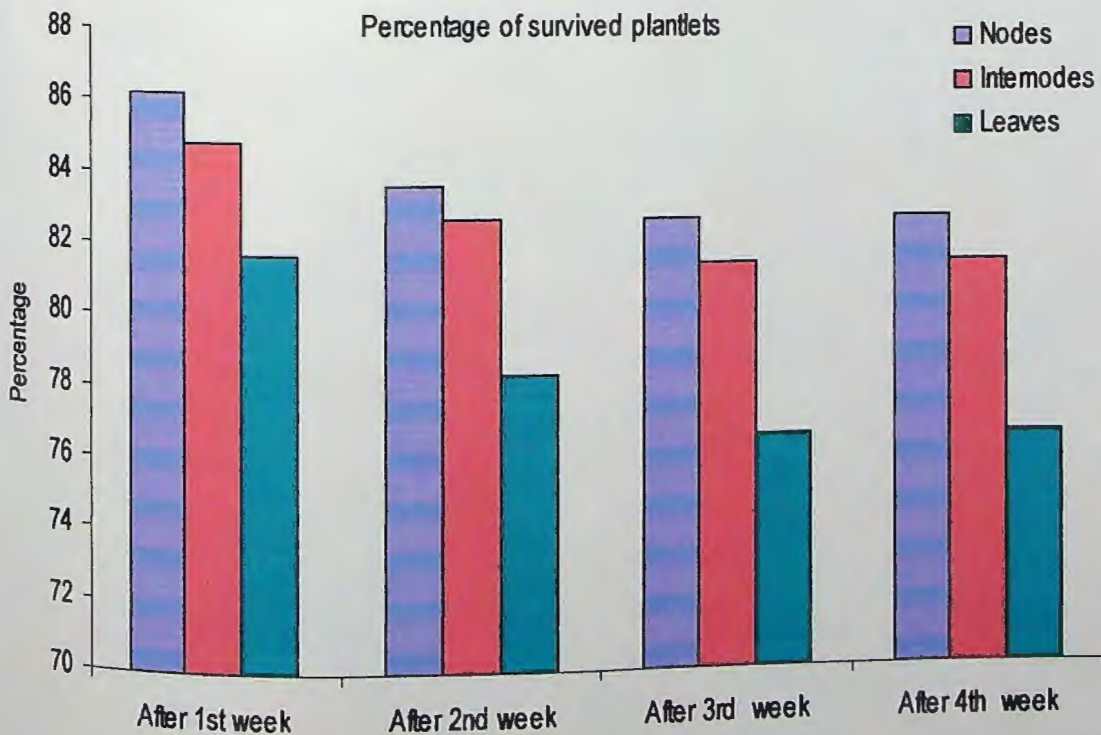
Fig. 41. Percentage of survived plantlets derived from the different explants of *Stevia rebaudiana* after transplantation under natural environment.

Plate 5. Establishment of plantlets of *Stevia rebaudiana* under natural environment
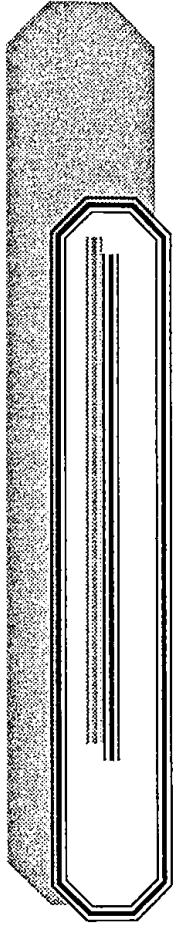
Chapter 4

Discussion

In *Stevia*, regeneration has been obtained by organogenesis from different explants: leaves (Ferreira and Handro 1987, 1988a, Yang and Chang 1979), axillary shoots (Bespalhok *et al*. 1995), stem tips (Tamura *et al*. 1984), suspension cultures (Ferreira and Handro 1988b), embryogenic callus (Bespalhok and Hattori 1997) and anthers (Flachsland *et al*. 1996).

In the present study the combination of BAP and NAA in the culture media was found the most effective on direct shoot proliferation from the different explants. In all cases nodes showed the highest percentage of proliferated shoots compared to internodes. BAP (2.0 mg/l) + NAA (0.2 mg/l) in the culture media showed the highest percentage of proliferated shoots from the nodes which was recorded 92.84 ± 0.45, average number of shoots per explant was 4.16 ± 0.28 and average length of the longest shoot was 2.67 ± 0.98 cm. Combination of BAP and NAA in the culture media was also found the most effective on indirect shoot proliferation from callus of the different explants. In all cases callus of nodes showed the highest percentage of proliferated shoots compared to the callus of internodes and leaves and BAP (2.0 mg/l) + NAA (0.2 mg/l) in the culture media was the best combination for the highest percentage of proliferated shoots from callus of all types of explants. Anbazhagan *et al*. (2010) cultured shoot tip, nodal segment and leaf as explants on Murashige and Skoog (MS) medium supplemented with different concentrations of BA, Kn and IAA both in individual and in combined form for shoot inductions and the best results were obtained from MS medium supplemented with BA+ IAA at the concentrations

of 1.0 mg/l and 0.5 mg/l respectively. Among the explants used, shoot tip stood first in inducing shoot development. Best root formation of *in vitro* developed shoots could be achieved on half-strength Nitsch (N6) medium supplemented with IAA at concentration 1.0 mg/l (Jena *et al.* 2009).

This *in vitro* propagation studies confirmed the importance of plant growth regulators in the initiations of callus, shoot, root and on the whole the regeneration of plant. In this way, the two cytokinins namely, BAP and Kn used in this study, the BAP proved a better one than Kn in shoot induction from all explants used i.e. nodes, internodes and leaves. Where as Kn could initiate shoot tip and nodal explants only and this is documented by early studies also (Murashige 1974, Benne and Davies 1986, Rogers *et al.* 1998). On the other hand, mineral nutrients are being as the basic component of culture media play a vital role in rapid growth of tissue and the extent and the quality of morphogenesis of tissue (Niedz and Evens 2007). In this study, the synergistic effect of both BAP + IAA at concentrations 2.0 mg/l and 0.2 mg/l respectively, were found best in regenerating shoot from shoot tip and nodal explants. Similar type of results have also been got by early workers in the same species, i.e. *S. rebaudiana* (Tamura *et al.* 1984, Ferreira and Handro 1988a,b, Patil *et al.* 1996, Sivaram and Mukundan 2003, Aparajita and Amita 2007, Ahmed *et al.* 2007, Mitra and Pal 2007, Jain *et al.* 2009, Patil and Shah 2009). *In vitro* propagation of *Stevia* has been carried out by Das *et al.* (2006) shows that 2, 4-D at 1.0 mg/l and kinetin at 0.2 mg/l combinations provide extensive initiation of callus which was proliferative and best maintained in NAA at 0.1mg/l and BAP at 2.0 mg/l combinations. For callus formation from the different explants, combination of 2,4-D and NAA was found the most effective. In all cases internodes showed the highest percentage of callus formation compared to nodes and leaves and 2,4-D (2.5 mg/l) + NAA (1.5 mg/l) in the culture media was the best combination for the highest percentage of callus formation from all types of explants.

NAA in the culture media was found the most effective on root proliferation from induced shoots of the different explants. In all cases induced shoots from nodes showed the

highest percentage of proliferated roots compared to induced shoots from internodes and leaves and NAA (0.2 mg/l) in the culture media was the best concentration for the highest percentage of proliferated roots from induced shoots of all types of explants. The root induction was gradually decreased with increasing concentrations of auxin types. Similar types of results were found by earlier workers in the same species (Sivaram and Mukundan 2003, Ahmed et al. 2007, Mitra and Pal 2007). Kalpana et al. (2010) incorporated a range of higher concentrations of $CuSO_4$-$5H_2O$ in MS medium significantly and fond enhanced direct shoot bud induction and proliferation from cultured leaf and nodal explants taken from mature plants of S. rebaudiana. Shoot bud induction medium was supplemented with BAP (2.2 µM) and NAA (2.8 µM). When the concentration of $CuSO_4$-$5H_2O$ in the induction medium was raised to 0.5 µM (five times the MS level, i.e. 0.1 µM) there was significant increase in percentage response along with increase in shoot bud number per explant. The shoots were healthy, well developed with dark green broader leaves. Ibrahim et al. (2008a) used nutrition medium MS at full salt strength of major and minor elements for in vitro culture of S. rebaudiana.

Ahmed et al. (2007) observed the induction of multiple shoots from nodal segments was the highest in MS medium supplemented with 1.5 mg/l BA + 0.5 mg/l Kn. For rooting different concentrations of IBA, NAA and IAA were used and highest rooting percentage (97.66%) was recorded on MS medium with 0.1 mg/l IAA. Taware et al. (2010) found optimal shoot initiation on medium containing 0.3 mg/l kinetin and root induction was optimized on MS medium with 2.0 mg/l IBA. Callus optimization was observed on MS medium supplemented with 0.1 mg/l 2,4-D; shoot initiation from callus was maximum in MS+BA+2,4-D. Uddin et al. (2006) made an explants culture of S. rebaudiana on MS medium containing 2,4-D at 2, 3, 4 and 5 mg/l for callus induction and observed the inter-nodal segments initiated callus earlier than node and leaf with the highest amount of callus was found with 3.0 mg/l 2,4-D. Rafiq et al. (2007) observed maximum shoot formation was by supplementing 2.0 mgl^{-1} BAP. In contrast 0.5 mgl^{-1} NAA caused the maximum root

formation in nodular stem sections of S. rebaudiana. Similar results were also observed by other workers in Bangladesh (Huda et al. 2007, Hossain et al. 2008, Ibrahim et al. 2008b).

Other researchers also reported the influence of cytokinin on shoot multiplications in different plant species. Bondarev (2001) reported that micro-cuttings with apical or axil buds were most effective explants for large-scale production, when cultivated on the hormone-free medium, and this allowed producing the plants by several thousand for 3-4 months. The addition of little concentrations of 6-BAP or α-NAA and 6-BAP to the nutrient medium was established to induce adventitious shoot formation by increasing a propagation coefficient (Sivaram and Mukundan (2003) reported the efficient regeneration of shoot apex, nodal, and leaf explants of S. rebaudiana when cultured on MS medium supplemented with 8.87 BAP and 5.71 µM IAA. The effect of NAA and IBA on root induction was positive, however, auxin's concentration had a positive effect on the number of roots/shoot and it was higher in the case of NAA than IBA Steven et al. (1992) reported that 1.0 mgl⁻¹ NAA in medium showed maximum rooting in regenerated shoots of S. rebaudiana. The potential of IBA in root induction bas been reported in many species. In root induction experiment, 0.5 mgl⁻¹ NAA was found better as maximum roots proliferated, that results are better than reported by Steven et al. (1992).

Kalpana et al. (2009) used liquid shoot culture of S. rebaudiana in MS medium containing BAP (1.5 mg/l) and IAA (0.5 mg/l) that developed and evaluated in relation to shoot multiplication, on average 37 new shoots per explants were obtained within 3 weeks. Direct shoot regeneration from Stevia leaves was done by Sreedhar et al. (2008)

Similar studies on shoot proliferation have been performed by Akita and Shigeoka (1994), Patil et al. (1996), Nepovin and Vanek (1998), Sikach (1998), Sivaram and Mukundan (2003) Ahmed et al. (2007), Debnath (2008) and Sairkar et al.(2009). They also reported that plant hormone is necessary for shooting, elongation and rooting. In most of cases BA was found to be essential for growth and multiple shoot formation.

The result of the experiment and other earlier research report clearly support the possibility of propagating *S. rebaudiana* by adopting *in vitro* techniques. The climatic requirements of this tropical elite medicinal plant indicate that it can be introduced in the hilly areas of Sylhet and Chittagong. The unique selling points of Stevia sweetener are very strong in Bangladesh due to the presence of diabetic and other metabolic disease including obesity. Here *Stevia in vitro* propagation has been demonstrated with its overall potentiality and suitability. *In vitro* propagation can become an important alternative to conventional propagation and breeding procedures for wide range of plant species.

The present investigation suggests that (i) *in vitro* micropropagation of *S. rebaudiana* may be the best way to bypass the limitation of conventional technique, (ii) MS medium with BAP (2.0 mg/l) was the superior to combination with NAA (0.2 mg/l) for shoot induction in micro cuttings, (iii) MS with NAA (0.2 mg/l) was the superior to IAA and IBA for days and length of root initiation in micro cutting. The culture established through the present investigation may also be commercially utilized as a source of disease free planting materials.
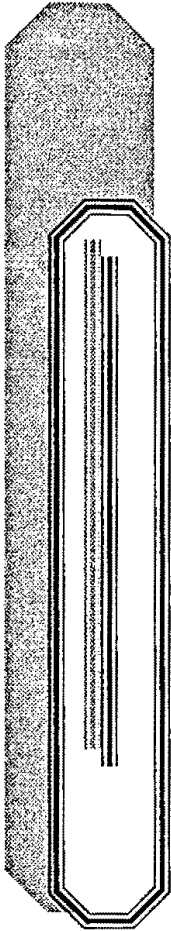
Chapter 5

Summary

SUMMARY

The present investigation was undertaken to standardize *in vitro* culture techniques for mass propagation and conservation of a non-sugar natural herbal sweetener, *Stevia rebaudiana* Bertoni. Nodes, internodes and leaves of pot grown mature plants were used as explants and experiments were based on surface sterilization; direct shoot proliferation; callus formation; indirect shoot proliferation from callus and root proliferation of induced shoot.

Surface sterilization of the different explants: For surface sterilization 0.1% $HgCl_2$ treatment for 2.5 minutes was found the most effect when nodes were used as explants, percentage of free contamination was 81.94 ± 0.94 and percentage of survived explants was 77.48 ± 0.35. $HgCl_2$ treatment for 2.5 minutes was also found the most effective for surface sterilization of internodes, percentage of free contamination was 84.43 ± 0.58 and percentage of survived explants was 79.83 ± 0.83. But in case of leaves, $HgCl_2$ treatment for 2.0 minutes was found the most effective, percentage of free contamination was 87.08 ± 1.27 and percentage of survived explants was 77.11 ± 1.11.

Direct shoot proliferation from the different explants: Combination of BAP and NAA in the culture media was found the most effective on direct shoot proliferation from the different explants. In all cases nodes showed the highest percentage of proliferated shoots compared to internodes. BAP (2.0 mg/l) +NAA (0.2 mg/l) in the culture media showed the highest percentage of proliferated shoots from the nodes which was recorded 92.84 ± 0.45, average number of shoots per explant was 4.16 ± 0.28 and average length of the longest shoot was 2.67 ± 0.98 cm. Highest percentage of proliferated shoots from the internodes

was recorded 84.87 ± 0.62 in the same combination of BAP and NAA in the culture media, average number of shoots per explant was 3.95 ± 0.34 and average length of the longest shoot was 2.78 ± 0.43 cm.

Callus formation from the different explants: For callus formation from the different explants, combination of 2,4-D and NAA was found the most effective. In all cases internodes showed the highest percentage of callus formation compared to nodes and leaves and 2,4-D (2.5 mg/l) + NAA (1.5 mg/l) in the culture media was the best combination for the highest percentage of callus formation from all types of explants. Highest percentage of callus formation was recorded 82.69 ± 1.40 from the internodes, average day of callus initiation was 10.00 ± 0.05 and colour of callus was green. In case of nodes, highest percentage of callus formation was recorded 70.34 ± 0.39, average day of callus initiation was 10.50 ± 0.15 and colour of callus was green. Again highest percentage of callus formation was recorded 68.21 ± 0.87 when leaves were used as explants, average day of callus initiation was 11.00 ± 0.15 and colour of callus was also green.

Indirect shoot proliferation from callus of the different explants: Combination of BAP and NAA in the culture media was found the most effective on indirect shoot proliferation from callus of the different explants. In all cases callus of nodes showed the highest percentage of proliferated shoots compared to the callus of internodes and leaves and BAP (2.0 mg/l) + NAA (0.2 mg/l) in the culture media was the best combination for the highest percentage of proliferated shoots from callus of all types of explants. Highest percentage of proliferated shoots from callus of the nodes which was recorded 94.70 ± 0.72, average number of shoots per callus was 4.25 ± 0.30 and average length of the longest shoot was 2.58 ± 0.12 cm. Highest percentage of proliferated shoots from callus the internodes was recorded 89.15 ± 1.05, average number of shoots per callus was 4.20 ± 0.16 and average length of the longest shoot was 2.73 ± 0.24 cm. Again highest percentage of proliferated shoots from callus of the leaves was recorded 84.73 ± 1.17,

average number of shoots per callus was 3.67 ± 0.42 and average length of the longest shoot was 2.57 ± 0.36cm.

Root proliferation from induced shoots of the different explants: NAA in the culture media was found the most effective on root proliferation from induced shoots of the different explants. In all cases induced shoots from nodes showed the highest percentage of proliferated roots compared to induced shoots from internodes and leaves and NAA (0.2 mg/l) in the culture media was the best concentration for the highest percentage of proliferated roots from induced shoots of all types of explants. Highest percentage of proliferated roots was recorded 93.33 ± 0.48 from induced shoots of the nodes, average number of roots per shoot was 10.40 ± 0.62 and average length of the longest root was 2.30 ± 0.15 cm. Highest percentage of proliferated roots from induced shoots of the internodes was 92.45 ± 0.72, average number of roots per shoot was 9.08 ± 0.28 and average length of the longest root was 2.38 ± 0.08 cm. Again highest percentage of proliferated roots was recorded 92.21 ± 0.33 from induced shoots of the leaves, average number of roots per shoot was 10.23 ± 0.55 and average length of the longest root was 2.36 ± 0.13 cm.

Establishment of plantlets derived from the different explants: Shoots with proliferated roots (new regenerated plantlets) were gradually acclimatized and established successfully on the soil under natural environment and percentages of survived plantlets from nodes, internodes and leaves were recorded 82.64%, 81.38%, and 76.53% respectively after 4th week of transplantation.

The methodologies developed in the present investigation are simple, highly effective and reproducible and can be utilized for in vitro mass propagation of Stevia rebaudiana Bertoni for its conservation and commercial exploitation.
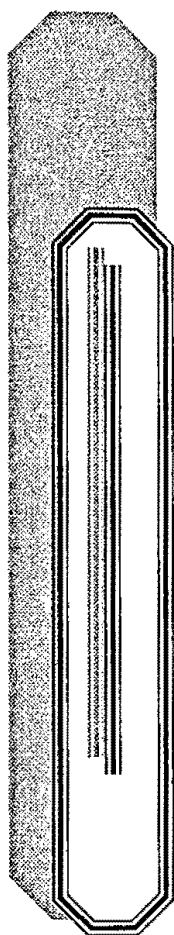
Chapter 6

Conclusion

Chapter 6

CONCLUSION

Significant progress has been made in the in vitro regeneration systems of many traditional medicinal plants. Using tissue culture protocols for the propagation of superior and/or endangered genotypes of medicinal plants, it is possible to produce healthy and disease-free plants which could be released to their natural habitat or cultivated on a large scale for the pharmaceutical product of interest. These are novel methods of conserving the natural populations of medicinal plants, reducing the risk of their extinction. *In vitro* propagation techniques impart vigor for the conservation process of the medicinal plants and also maintain the clonal uniformity not achieved by using seeds. Various strategies for using in vitro systems are being studied extensively with the objective of improving the production and qualitative consistency of plant chemicals. Due to these advances, research in the area of tissue culture technology for production of plant chemicals has bloomed beyond expectations.

The results of the experiment and earlier research reviews clearly support the initiation and maintenance of callus of *Stevia rebaudiana* by adopting *in vitro* techniques. *In vitro* propagation of this plant has been carried out with its overall potentiality and suitability using different phytohormone combinations and the results, show that Cytokinin (BAP) and Auxin (NAA) combination was better for both direct and indirect shoot proliferation, 2,4-D and NAA combination was better for callus initiation and for callus maintainance, and NAA was better for root proliferation from the induced shoots. By using the method described

above, hundreds of clonal plants can be produced from one nodal explant by continuous subculturing of shoot propagules. The multiplication rate that was achieved was not significantly large to be commercially significant, but the results provide a basis for further research in micropropagation of other genotypes of *S. rebaudiana*.
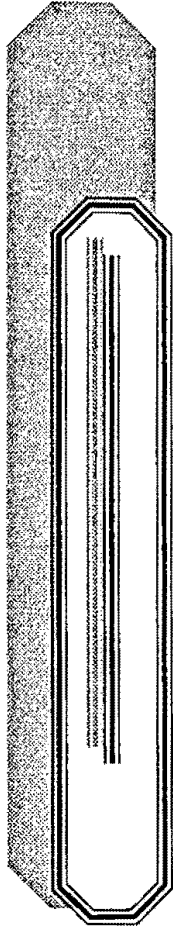
Chapter 7

References

REFERENCES

Ahmed M B, Salahin M, Karim R, Razvy M A, Hannan M M, Sultana R, Hossain M and Islam R 2007. An efficient method for *in vitro* clonal propagation of a newly introduced sweetener plant (*Stevia rebaudiana*) in Bangladesh. *American-Eurasian J. Sci. Res.* 2(2): 121-125.

Ahmed M J and Smith R M 2002. Determination of stevioside by high performance liquid chromatography with pulsed amperometric detection. *J Separation* Sci., 25: 170-172.

Akerele O 1992. WHO guidelines for the assessment of herbal medicine. *Fitoterapia* **62**(2): 99-110.

Akita M and Shigeoka T 1994. Mass propagation of shoots of stevia rebaudiana using a large scale bioreactor. *Plant Cell Rep.* 13(3-4): 180-183.

Anbazhagan M, Kalpana M, Rajendran R, Natarajan V and Dhanavel D 2010. *In vitro* production of *Stevia rebaudiana* Bertoni. *Emir. J. Food Agric.* 22 (3): 216-222

Anonymous 1999. Opinion on *Stevia rebaudiana* Bertoni plants and leaves http://ec. europa.eu/food/fs/sc/scf/out36_en.pdf (Accessed on 23/04/07).

Aparajita M and Amita P 2007. *In vitro* regeneration of *Stevia rebaudiana* (bert) from the nodal explant. *J. Plant Biochem. Biotechnol.*, 16(1): 71-72.

Bajaj Y P S 1995. Cryopreservation of germplasm of medicinal and aromatic plants. In: Bajaj Y P S [ed.] *Biotechnology in agriculture and forestry, cryopreservation of plant germplasm* I, vol. 32, pp. 419–434, Springer-Verlag; Berlin.

Benne L K and Davies F T 1986. In vitro propagation of *Quercus shumardii* seedling. *Hort. Sci.* 21: 1045-1047.

Bespalhok J C, and Hattori K 1997. Embryogenic callus formation and histological studies from *Stevia rebaudiana* (Bert.) Bertoni floret explant. *R. Bras. de Fisiol. Veg.*, 9(3): 185-188.

Bespalhok J C, Hashimoto J M and Vieira L G E 1995. Induction of somatic embryogenesis from leaf explants of *Stevia rebaudiana*. *R. Bras. de Fisiol. Veg.*, 5(1): 51-53.

Bhojwani S S and Razan M K 1996. Clonal Propagation. In: Bhojwani S S and Razan M K (Eds.). *Plant Tissue Culture: Theory and Practice*, A Revised Edition. Elsevier, Amsterdam. pp. 483-536.

Bodeker G 2002. Medicinal plants: towards sustainability and security. Discussion paper for MEDPLANT. Available at website http://source.bellanet.org/ medplant/docs /ssong/ MEDPLANT_Discussion_Paper1.doc.

Bondarev N 2001. Peculiarities of propagation and development of *Stevia rebaudiana* Bertoni plants *in vitro*. *Proc. 9th Int. Conf. Hort.*, September 3-6, 2001. Lednice, Czech Republic, 2: 431-434.

Bramwell D 1990. The role of in vitro cultivation in the conservation of endangered species. In: Hernández Bermejo J E, Clemente M, Heywood Y [eds.], *Conservation techniques in botanic gardens*, pp. 3-15, Koeitz Scientific Books, Koenigstein.

Brandle J E and Rosa N 1992. Heritability for yield, leaf:stem ratio and stevioside content estimated from a landrace cultivar of *Stevia rebaudiana*. *Can. J. Plant Sci.* **72**: 1263-1266.

Bridel M and Lavieille R 1931a. Le principe à saveur sucrée du Kaà-hê-é (*Stevia rebaudiana)* Bertoni. *Bull. Soc. Chim. Biol.* **13**: 636-655.

Bridel M and Lavieille R 1931b. Le principe sucré du Kaà-hê-é (*Stevia rebaudiana* Bertoni). II. L'hydrolyse diastasique du stévioside. III. Le stéviol de l'hydrolyse diastasique et l'isostéviol de l'hydrolyse acide. *Bull. Soc. Chim. Biol.* **13**: 781-796.

Carneiro J W P 1990. *Stevia rebaudiana* (Bert.) Bertoni, production of seed. M.Sc Thesis, State University of Maringa, Brazil (English abstr.).

Caswell K L, Leung N and Chibbar R N 2000. An efficient method for *in vitro* regeneration from immature inflorescence explants of Canadian wheat cultivars. *Plant cell Tissue organ Cult.*, 60: 69-73.

Chang S S and Cook J M 1983. Stability studies of stevioside and rebaudioside A in carbonated beverages. J. *Agric. Food Chem.* **31**: 409-412.

Chang W D, Gau T G, Chang D D, Chern Y C, Chen C C, Yeh F T, Chang Y S, Hsieh M T, Chu N Y and Tsay H S 1992. Rapid clonal propagation and production of useful compounds of Chinese medicinal herbs by tissue and cell suspension culture. In. *Proc. of the SABRAO Int. Symp.* March 10–13, pp. 207-211, The impact of biological research on agricultural productivity, National Chung-Hsing University Taichung, Taiwan,

Chang W D, Huang W W, Chen C C, Chang Y S and Tsay H S 1994. The production of secondary metabolites from Chinese medicinal herbs by suspension cell and tissue culture. In. *Proc. 7th Int. Congr of SABRAO and WASS*, November 16–19, pp. 535-540, Academia Sinica, Taipei, Taiwan.

Constabel F 1990. Medicinal plant biotechnology. *Planta Med.* **56**: 421–425.

Cook I F and Knox J R 1970. A synthesis of steviol. *Tetrahedron Lett.* 4091-4093.

Crammer B. and Ikan R 1986. Sweet glycosides from the stevia plant. *Chem. Brit.* **22**: 915-916.

Cunningham A B 1993. African medicinal plants: setting priorities at the interface between conservation and primary health care. People and plant initiative working paper 1. Nairobi: UNESCO, http://www.rbgkeworg.uk/peopleplant/wp/wp1/index.htm.

Darise, M., Kohda, H., Mizutani, K., Kasai, R. and Tanaka, O. 1983. Chemical constituents of flowers of *Stevia rebaudiana* Bertoni. *Agric. Biol. Chem.* **47**: 133-135.

Das K, Dang R and Rajasekharan P E 2006. Establishment and maintenance of callus of *Stevia rebaudiana* Bertoni under aseptic environment. *Nat. Prod. Radiance*, 5(5): 373-376.

De Oliveira V M, Forni-Martins E R, Magalhães P M and Marcos N A 2004. Chromosomal and morphological studies of diploid and polyploid cytotypes of *Stevia rebaudiana* Bertoni (Eupatorieae, Asteraceae). *Genet. Mol. Biol.*, 27: 215-222.

Debergh P C and Maene L J 1981. A scheme for commercial propagation of ornamental plants by tissue culture. *Sci. Hort.* **14**: 335–345.

Debnath M 2008. Clonal propagation and antimicrobial activity of an endemic medicinal plant *Stevia rebaudiana*. *J. Med. Plants Res.* 2(2): 45-51.

Delporte F, Mostade O and Jacquemin J M 2001. Plant regeneration through callus initiation from thin mature embryo fragments of wheat. *Plant Cell Tissue Organ Cult.*, 67: 73-80.

Djerassi C, Quitt P, Mosettig E, Cambie R C, Rutledge P S and Briggs L H 1961. Optical rotatory dispersion studies. LVIII. The complete absolute configurations of steviol, kaurene and the diterpene alkaloids of the garryfoline and atisine groups. *J. Amer. Chem. Soc.* **83**: 3720-3722.

Dolder F, Lichti H, Mosettig E and Quitt P 1960. The structure and stereochemistry of steviol and isosteviol. *J. Amer. Chem. Soc.* **82**: 246-247.

Donalisio M G R, Duarte F R, Pinto A J D A and Souza C J 1982. *Stevia rebaudiana*. *Agronomico* **34**: 65-68.

Dubois G E and Stephenson R A 1984. Diterpenoid sweeteners. Synthesis and sensory evaluation of stevioside analogues with improved organoleptic properties. *J. Med. Chem.* **28**:93-98.

Fay M F 1992. Conservation of rare and endangered plants using *in vitro* methods. *In Vitro Cell. Dev. Biol. Plant* **28**: 1–4.

Felippe G M and Lucas N M C 1971. Estudo da viabili dade dos fructosde *Stevia rebaudiana* Bert. *Hoehnea* 1: 95–105.

Ferreira C M and Handro W 1987. Some morphogenetic responses of leaf explants of *Stevia rebaudiana* cultured *in vitro*. *R. Bras. de Bot.*, 10: 113-116.

Ferreira C M and Handro W 1988a. Micropropagation of *Stevia rebaudiana* through leaf explants from adult plants. *Planta Med.*, 54:157-160.

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

Flachsland E, Mroginski L and Daviña J 1996. Regeneration of plants from anthers of *Stevia rebaudiana* Bertoni (Compositae) cultivated *in vitro*. *Biocell*, 20:87-90.

Fors A 1995. A new character in the sweetener scenario. *Sugar J.* **58**: 30.

Frederico A P, Ruas P M, Marinmorlaes M A, Ruas C F and Nakajima J N 1996. Chromosome studies in some *Stevia* (Compositae) species from southern Brazil. *Braz. J. Genet.* **19**: 605-609.

Fujita S, Taka K and Fujita Y 1977. Miscellaneous contributions to the essential oils of the plants from various territories. XLI. The components of the essential oil of *Stevia rebaudiana*. *Yakugaku Zasshi* 97: 692-694; *Chem. Abstr.* **87**: 122621v (1977).

Gentry A H 1996. A Field Guide of the Families and Genera of Woody Plants of Northwest South America (Colombia, Ecuador, Peru) with Supplementary Notes on Herbaceus Taxa. The University of Chicago Press, Chicago, 895 pp.

George E F and Sherrington P D 1984. *Plant propagation by tissue culture*. Exegetics Ltd. London.

Ghosh S, Subudhi E and Nayak S. 2008. Antimicrobial assay of Stevia *rebaudiana* Bertoni leaf extracts against 10 pathogens. *Int. J. Integratve Biol.* 2(1): 27-31.

Goenadi D H 1983. Water tension and fertilization of *Stevia rebaudiana* Bertoni on Oxic Tropudalf (English abstr.). *Menara Perkebunan.* **51**: 85-90.

Goettemoeller and Ching 1999. Seed germination of Stevia rebaudiana : Perspective on new crops and new uses. Janick J (ed), ASHS Press, Alexandria, VA. Pp510-511.

Haebisch E M A B 1992. Pharmacological trial of a concentrated crude extract of Stevia rebaudiana (Bert.) Bertoni in healthy volunteers. Arq. Biol. Technol. 35:299-314.

Handro W and Ferreira C M 1989. Chromosomal variability and growth rate in cell suspension cultures of Stevia rebaudiana Bertoni. Plant Sci. Limerick 93(1-2): 169-176.

Hanson J R and De Oliveira B H 1993. Stevioside and related sweet diterpenoid glycosides. Nat. Prod. Rep. 10: 301-309.

Hoareau L and DaSilva E 1999. Medicinal plants: a re-emerging health aid. Electronic J. Biotech. 2: 56-70.

Hossain M A, Kabir A H M S, Jahan T A and Hasan M N. 2008. Micropopagation of Stevia. Int. J. Sustain. Crop Prod. 3(4):1-9

Huda M N, Ahmed A, Mandal C, Alam K A, Reza M S H and W A. 2007. In vitro morphogenic responses of different explants of Stevia (Stevia rebaudiana Bert.). Int. J. Agri. Res. 2(12): 1006-1013.

Ibrahim I A, Nasr M I, Mohammed B R and El-Zefzafi 2008a. Plant growth regulators affecting in vitro cultivation of Stevia rebaudiana. Sugar Tech. 10(3): 248-253.

Ibrahim I A, Nasr M I, Mohammed B R and El-Zefzafi 2008b. Nutritional factors affecting in vitro cultivation of Stevia rebaudiana. Sugar Tech. 10(3): 254-259.

Ibrahim N A, El-Gengaihi S, Motawe H and Riad S A 2007. Phytochemical and biological investigation of Stevia rebaudiana Bertoni; 1-labdane-type diterpene. Eur. Food Res. Technol., 224: 483-488.

Ishima N and Katayama O 1976. Sensory evolution of stevioside as a sweetener. Rep. Natl. Fwd. Resp. Inst. 31: 80-85.

Itagaki K and Ito T 1979. Purification of stevioside (English abstr.). Jap. Patent 54-041898.

Jain P, Kachhwaha S and Kothari S L 2009. Improved micro propagation protocol and enhancement in biomass and chlorophyll content in *Stevia rebaudiana* Bertoni by using high copper levels in the culture medium, *Sci. Hort.* 119: 315-319.

Jayaraman S, Manoharan, M S, and Illanchezian S 2008. In-vitro Antimicrobial and Antitumor Activities of *Stevia rebaudiana* (Asteraceae) Leaf Extracts. *Trop. J. Phar. Res.* 7 (4): 1143-1149

Jena A K, Panda M K and Nayak S 2009. Effect of phytoregulators on *in vitro* mass propagation of *Stevia rebaudiana* Bertoni. *Int. J. Integra. Biol.*, 8(1): 56-59.

Jeppensen P B, Gregersen S, Rolfsen S E D, Jepsen M, Colombo M, Agger A, Xiao J, Kruhøffer M. Ørntoft T and Hermansen K. 2003. Antihyperglycemic and blood pressure-reducing effects of Stevioside in the diabetic Goto-Kakizaki Rat. *Metabolism*, 52: 372-378.

Kalpana M, Anbazhagan M and Natarajan V 2009. Utilization of liquid medium for rapid micropropagation of *Stevia rebaudiana* Bertoni. *J. Ecobiotechnol.* 1(1): 16-20

Kalpana M, Anbazhagan M, Natarajan V and Dhanavel D. 2010. Improved micropropagation method for the enhancement of biomass in *Stevia rebaudiana* Bertoni . *Recent Res. Sci. Technol.*, 2(1): 8-13

Kaneda N, Kasai R, Yamasaki K and Tanaka O 1977. Chemical studies on sweet diterpene-glycosides of *Stevia rebaudiana*: conversion of stevioside into rebaudioside-A. *Chem. Pharm. Bull.* 25: 2466-2467.

Katayama O, Sumida T, Hayashi H and Mitsuhashi H 1976. The practical application of Stevia and research and development data (English translation). I.S.U. Company, Japan. 747 pp.

Kim N C and Kinghorn A D 2002. Highly sweet compounds of plant origin. *Arch. Pharm. Res.* 25: 725-746.

Kinghorn A D and Soejarto D D 1985. Current status of stevioside as a sweetening agent for human use. In: Wagner H, Hikino H and Farnsworth N R [eds.] *Economic and medicinal plant research*, pp. 1-52. Academic Press, London.

Kobayashi M, Horikawa S, Degrandi I H, Ueno J and Mitsuhashi H 1977. Dulcosides A and B, new diterpene glycosides from *Stevia rebaudiana*. *Phytochemistry* **16**: 1405-1408.

Kohda H, Kasai R, Yamasaki K, Murakami K and Tanaka O 1976. New sweet diterpene glucosides from *Stevia rebaudiana*. *Phytochemistry* **15**: 981-983.

Koyama E, Kitazawa K, Ohori Y, Izawa O, Kakegawa K and Fujino A 2003. *In vitro* metabolism of the glycosidic sweeteners, stevia mixture and enzymatically modified stevia in human intestinal microflora. *Food Chem. Toxicol.* 41: 359–374.

Lee J I, Kang K K. and Lee E U 1979. Studies on new sweetening resource plant stevia (*Stevia rebaudiana* Bert.) in Korea. I. Effects of transplanting date shifting by cutting and seeding dates on agronomic characteristics and dry leaf yields of stevia (English abstr.). *Res. Rep. ORD* **21**: 171-179.

Lee J I, Kang K H, Park H W and Ham Y S 1982. New high rebaudioside - A stevia variety "Suweon 11" (English abstr.). *Res. Rep. ORD* **24**: 186-188.

Lester T. 1999. *Stevia rebaudiana* (Sweet Honey Leaf). *The Aust. New Crop Newsl.* 11

Lewington A 1993. Medicinal plants and plant extracts: a review of their importation into Europe. A traffic network report. Cambridge: Traffic International.

Lewis W H 1992. Early uses of *Stevia rebaudiana* (Asteraceae) leaves as a sweetener in Paraguay. *Econ. Bot.* **46**: 336-337.

Mantovaneli I C C, Ferretti E C, Simões M R and Da Silva F C 2004. The effect of temperature and flow rate on the clarification of the aqueous stevia-extract in a fixed-bed column with zeolites. *Brazilian J. Chemical Eng.*, 21: 449 – 458.

Markovic I S, Darmati Z A and Abramovic B F 2008. Chemical composition of leaf extracts of *Stevia rebaudiana* Bertoni grown experimentally in Vojvodina. *J, Serb. Chem, Soc.* 73(3): 283-297.

Martelli A, Frattini C and Chialva F 1985. Unusual essential oils with aromatic properties-I. Volatile components of *Stevia rebaudiana* Bertoni. *Flav. Frag. J.* 1: 3-7.

Matsushita K and Kitahara T 1981 Separation of stevioside and rebaudioside A by crystallization (English abstr.). *Jap. Patent* 56-121454.

McGarvey B D, Attygalle A B, Starratt A N, Xiang B, Schroeder F C, Brandle J E and Meinwald J 2003. New non-glycosidic diterpenes from the Leaves of *Stevia rebaudiana. J. Natural Product,* 66: 1395-1398.

Melis M S 1992a. Influence of calcium on the blood pressure and renal effects of stevioside. *Braz. J. Med. Biol. Res.* 25: 943-949.

Melis M S 1992b. Stevioside effect on renal function of normal and hypertensive rats. *J. Ethnopharmacol.,* 36: 213-217.

Melis M S. 1999. Effect of chronic administration of *Stevia rebaudiana* on fertility in rats. *J. Ethnophar.* 67: 157-161.

Melis M S and Sainati A R 1991. Effect of calcium and verapamil on renal function of rats during treatment with stevioside. *J. Ethnopharmacol.,* 33: 257-262.

Mendelsohn R and Balick M 1994. The value of undiscovered pharmaceuticals in tropical forests. *Econ. Bot.* 49: 223-228.

Metivier J and Viana A M 1979. Determination of microgram quantities of stevioside from leaves of *Stevia rebaudiana* Bert. by two-dimensional thin layer chromatography. *J. Exp. Bot.* 30: 805-810.

Midmore D J and Rank A H 2002. A new rural industry- Stevia to replace imported chemical sweeteners, RIRDC web publication, Project No. UCQ- 16A. www.rirdc.gov.au

Mitra A and Pal A 2007. *In vitro* regeneration of *Stevia rebaudiana* (Bert.) from nodal explants. *J. Plant Biochem. Biotechnol.* 16:59-62.

Miura, Y.; Fukui, H. and Tabata, M. 1987. Clonal propagation of chemically uniform fennel plants through somatic embryoids. *Planta Med.* 53: 92-94.

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 Med.* 52: 321-323.

Miyazaki Y and Watanabe H 1974. Studies on the cultivation of *Stevia rebaudiana* Bertoni; On the propogation of the plant (English abstr.). *Jap. J. Trop. Agric.* **17**: 154-157.

Mori K, Nakahara Y and Matsui M 1972. Diterpenoid total synthesis-XIX. (±)-Steviol and erythroxydiol A: rearrangements in bicyclooctane compounds. *Tetrahedron* **28**: 3217-3226.

Morita T 1987. Dried leaves (English abstr.). Jap. Patent 62-96025

Mosettig E and Nes W R 1955. Stevioside. II. The structure of the aglucone. *J. Org. Chem.* **20**: 884-899.

Mosettig E, Beglinger U, Dolder F, Lichti H, Quitt P and Waters J A 1963. The absolute configuration of steviol and isosteviol. *J. Amer. Chem. Soc.* **85**: 2305-2309.

Murai N 1988. Stevia drying system (English abstr.) Jap. Patent 63-258553.

Murashige T 1974. Plant propagation through tissue culture. *Annu. Rev. Plant Physiol.*, 25:135–166.

Murashige T and Skoog F 1962. A revised medium for rapid growth and bioassay with tobacco tissue cultures. *Physiol. Plant* 15, 473–497.

Murch S J, KrishnaRaj S and Saxena P K 2000. Phyto-pharmaceuticals: massproduction, standardization, and conservation. *Herbal Med.* 4(2): 39–43.

Nabeta K, Kasai T and Sugisawa H 1976. Phytosterol from the callus of *Stevia rebaudiana*. *Agric. Biol. Chem.* **40**: 2103-2104.

Naik G R 1998. Micropropagation studies in medicinal and aromatic plants. In: Khan I A, Khanun A [eds.] *Role of biotechnology in medicinal and aromatic plants*, pp. 50-56, Ukaaz Publications, Hyderabad, India.

Nakahara Y, Mori K. and Matsui M. 1971. Diterpenoid total synthesis. Part XVI. Alternative synthetic routes to (±)-steviol and (±)-kaur-16-en-19-oic acid. *Agric. Biol. Chem.* **35**: 918-928.

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

Nalawade S M, Sagare A P, Lee C Y, Kao C L and Tsay H S 2003. Studies on tissue culture of Chinese medicinal plant resources in Taiwan and their sustainable utilization. *Bot. Bull. Acad. Sin.* **44**: 79–98.

Narayanaswamy S 1977. Regeneration of plants from tissue cultures. In: Reinert, J.; Bajaji, Y.P.S. [eds.] *Applied and fundamental aspects of plant cell, tissue and organ culture*, pp. 179-248. Springer-Verlag; New York.

Nepovim A and Vanek T 1998. *In vitro* propagation of *Stevia rebaudiana* plants using multiple shoot culture. *Planta Medica* 64(8): 589-593.

Niedz R P and Evens T J 2007. Regulating plant tissue growth by mineral nutrition. *In Vitro Cell. Dev. Biol. Plant* 43: 370–381.

Ogawa T 1980. Decolorization and purification of a stevia sweet component (English abstr.). *Jap. Patent* 55-111768.

Ogawa T, Nozaki M and Matsui M 1980. Total synthesis of stevioside. *Tetrahedron* **36**: 2641-2648.

Oshima Y, Saito J and Hikino H 1986. Sterebins A, B, C and D, bisnorditerpenoids of *Stevia rebaudiana* leaves. *Tetrahedron* **42**: 6443-6446.

Oshima Y, Saito J and Hikino H 1988. Sterebins E, F, G and H, diterpenoids of *Stevia rebaudiana* leaves. *Phytochemistry* **27**: 624-626.

Pandiyan R, Velu1 R K, Chandrasekaran S K and Bashiyam P 2009. Effect on extracts of *Stevia rebaudiana* Bertoni in ethanol induced gastric ulcer by using Wister rats. Recent Res. Sci. Technol. 1(3): 127–130

Park J-E and Cha Y-S 2010. *Stevia rebaudiana* Bertoni extract supplementation improves lipid and carnitine profiles in C57BL/6J mice fed a high-fat diet. *J. Sci. Food Agric.* 90: 1099–1105

Patel R M and Shah R R 2009. Regeneration of stevia plant through callus culture. *Indian J. Pharm. Sci.*, 71:46-50

Patil V, Reddy P C, Purushotham M G, Prasad T G and Udayakumar M 1996. *In vitro* multiplication of *Stevia rebaudiana. Curr. Sci.* 70(11): 960.

Phillips K C 1989. Stevia: steps in developing a new sweetener. In: Grenby T H [ed.] *Developments in sweeteners*, Volume 3. pp. 1-43. Elsevier Applied Science, London.

Preece J E and Sutter E G 1991. Acclimatization of micropropagated plants to green house and field. In: Debergh P C, Zimmerman R H [eds.] *Micropropagation technology and application*, pp. 71-93. Kluwer Academic, Boston.

Rafiq M, Dahot M U, Mangrio S M, Naqvi H A and Qarshi I A. 2007. *In vitro* clonal propagation and biochemical analysis of field established *Stevia rebaudiana* Bertoni. *Pak. J. Bot.*, 39(7): 2467-2474

Rajbhandari A and Roberts M F 1983. The flavonoids of *Stevia rebaudiana*. *J. Nat. Prod.* **46**: 194-195.

Rajesh P, Khannan V R and Duran M T 2010. Effect of *Stevia rebaudiana* Bertoni ethanolic extract on anti-cancer activity of Erlisch's Ascites carcinoma induced mice. *Current Biota,* 3(4): 549-554.

Ramage C M and Willams R R 2002. Mineral nutrition and plant morphogenesis. *InVitro Cell. Dev. Biol.-Plant.* 38: 116–124.

Read P E 1988. Stock plant influence micropropagation success. *Acta. Hort.* **226**: 41–52.

Rendle A B 1979. *The classification of flowering plants.* Cambridge University Press, Vol. II, India, pp. 587.

Robinson B L 1930. Contributions from the Gray Herbarium of Harvard University. The Gray Herbarium of Harvard University, Cambridge.

Rogers D S, Beech J and Sharma K S 1998. Shoot regeneration and plant acclimatization of the wetland monocot Cattail (*Typha latifolia*). *Plant Cell Rep.* 18: 71–75.

Rojas C-G and Miranda P 2002. HPLC isolation and structural elucidation of diastereomeric niloyl ester tetrasachharides from Mexican scammony root. *J. Tetrahedron,* 58:3145-3154.

Rout G R, Samantaray S and Das P 2000. *In vitro* manipulation and propagation of medicinal plants. *Biotechnol. Adv.* **18**: 91–120.

Chapter 7 ᒫ References

Sagare A P, Kuo C L, Chueh F S and Tsay H S 2001. *De novo* regeneration of *Scrophularia yoshimurae* Yamazaki (Scrophulariaceae) and quantitative analysis of harpagoside, an iridoid glucoside, formed in aerial and underground parts of *in vitro* propagated and wild plants by HPLC. *Biol. Pharm. Bull.* **24**: 1311–1315;

Sairkar P, Chandravanshi M K, Shukla N P and Mehrotra N N 2009. Mass production of an economically important medicinal plant *Stevia rebaudiana* using *in vitro* propagation techniques. *J. Med. Plants Res.* 3(4): 266-270

Sajc L, Grubisic D and Vunjak-Novakovic G 2000. Bioreactors for plant engineering: an outlook for further research. *Biochem. Engng. J.* **4**: 89–99.

Sakaguchi M and Kan T. 1982. Japanese research on *Stevia rebaudiana,Ci. Cult.* 34:235-248.

Sakamoto I, Yamasaki K and Tanaka O 1977a. Application of ^{13}C NMR spectroscopy to chemistry of natural glycosides: rebaudioside-C, a new sweet diterpene glycoside of *Stevia rebaudiana. Chem. Pharm. Bull.* **25**: 844-846.

Sakamoto I, Yamasaki K and Tanaka O 1977b. Application of ^{13}C NMR spectroscopy to chemistry of plant glycosides: rebaudiosides-D and -E, new sweet diterpene-glucosides of *Stevia rebaudiana* Bertoni. *Chem. Pharm. Bull.* **25**: 3437-3439.

Savita S M, Sheela K, Sunanda S, Shankar A G, Ramakrishna P and Sakey S 2004. Health implications of *Stevia rebaudiana. J. Human Ecol.*, 15: 191-194.

Saxena N C and Ming I S 1988.Preliminary harvesting characteristics of *Stevia. Phys. Prop. Agic. Mat. Prod.* 3: 299-303.

Schiffman S S, Booth B J, Carr B T, Losee M L, Sattely-Miller E and Graham B G 1995. Investigation of synergism in binary mixtures of sweeteners. *Brain Res. Bull.* **38**: 105-120.

Schiffman S S, Pecore S D, Booth B J, Losee M L, Carr B T, Sattely-Miller E, Graham B G and Warwick Z S 1994. Adaptation of sweeteners in water and in tannic acid solutions. *Phys. Behavior* **55**: 547-559.

Schock, C C 1982. Experimental cultivation of Rebaudi's stevia in California. University of California Agronomy Progress Report No. 122.

Shaffert E E and Chetobar A A 1992. Development of the male gametophyte in *Stevia rebaudiana* (English abstr.). *Buletinul Academiei de Schtintse A Republica Moldava* **6**: 3-9.

Shaffert E E and Chetobar A A 1994a. Development of the female gametophyte in *Stevia rebaudiana* (English translation). *Buletinul Academiei de Schtintse A Republica Moldava* **6**: 10-18.

Shaffert E E and Chetobar A A 1994b. Structure, topography and onogeny of *Stevia rebaudiana* (English abstr.). *Botanicheskii Zhurnal* **79**: 38-48.

Shaharan V, Yadav RC, Yadav N R and Chapagain B P 2004. High frequency plant regeneration from desiccated calli of *indica rice (Oryza sativa L.)*. *Afr. J. Biotechnol.*, 3:256-259.

Shibata H, Sonoke S, Ochiai H, Nishihashi H and Yamada M 1991. Glycosylation of steviol and steviol-glucosides in extracts from *Stevia rebaudiana* Bertoni. *Plant Physiol.* **95**: 152-156.

Shimomura K, Yoshimatsu K, Jaziri M and Ishimaru K 1997. Traditional medicinal plant genetic resources and biotechnology applications. In: Watanabe, K.; Pehu, E.R.G. [eds.] *Plant biotechnology and plant genetic resources for sustainability and productivity*, pp. 209-225. R.G. Landes Company and Academic Press Inc.; Austin, TX.

Shizhen S 1995. A study on good variety selection in *Stevia rebaudiana*. *Scientia Agricultura Sinica* **28**: 37-41.

Shock C C 1982. Experimental cultivation of Rebaudia's *Stevia* in California. University of California Agronomy Progress Report No. 122.

Sholichin M, Yamasaki K, Miyama R, Yahara S and Tanaka O 1980. Labdane-type diterpenes from *Stevia rebaudiana*. *Phytochemistry* **19**: 326-327.

Shuping C and Shizhen S 1995. Study on storage technique of *Stevia rebaudiana* seed (English abstr.). *Acta Agronomica Sinica* 21: 102-105.

Shyu Y T, Liu S Y, Lu H Y, Wu W K and Su C G 1994. Effects of harvesting dates on the charateristics, yield and sweet components of stevia (*Stevia rebaudiana* Bertoni) lines. *J. Agric. Res. China* 43:29-39.

Sikach V O 1998. Effect of nutrient media on physiological peculiarities of *Stevia rebaudiana* plants cultivated *in vitro*. *Fiziologiya Bikhimiya Kulturnykh Rastenii* 30(4): 294-299.

Sivaram L and Mukundan U 2003. *In vitro* culture studies on Stevia rebaudiana. *In Vitro Cell. Dev. Biol.: Plant,* 39(5): 520-523.

Soejarto D D, Kinghorn A D and Farnsworth N R 1982. Potential sweetening agents of plant origin. III. Organoleptic evaluation of stevia leaf herbarium samples for sweetness. *J. Nat. Prod.* 45: 590-599.

Soejarto D D, Compadre C M, Medon P J, Kamath S K and Kinghorn A D 1983. Potential sweetening agents of plant origin. II. Field search for sweet-tasting Stevia species. *Econ. Bot.* 37: 71-79.

Sreedhar R V, Venkatachalam L, Thimmaraju R, Bhagyalakshmi N, Narayan M S and Ravishankar G A 2008. Direct organogenesis from leaf explants of *Stevia rebaudiana* and cultivation in bioreactor. *Biologia Plantarum,* 52(2): 355-360.

Srivastava J, Lambert J and Vietmeyer N 1995. Medicinal plants: an expanding role in development. World Bank technical paper no. 320. Washington, DC: World Bank Agriculture and Forestry Systems.

Starrat A N, Kirby C W, Pocs R and Brandle J E 2002. Rebaudioside F, a diterpene glycoside from Stevia rebaudiana. *Phytochemistry,* 59: 367–370.

Steel R G D and Torrie J H 1982. *Principles and Procedures of Statistics.* 2nd Ed., McGraw Hill Book Co., Tokyo, Japan.

Steven M S, Gail, B M and Christopher W W B 1992. Stevioside biosynthesis by callus, root, shoot and rooted-shoot cultures. *In vitro. Plant Cell, Tiss Org.,* 28:151-158.

Sumida T 1968. Reports on *Stevia rebaudiana* Bertoni M. introduced from Brazil as a new sweetness resource in Japan. *Misc. Pub. Hokkaido Natl. Exp. Sta.* **2**: 69-83.

Sumida T 1980. Studies on *Stevia rebaudiana* Bertoni as a new possible crop for sweetening resource in Japan (English summary). *J. Cent. Agric. Exp. Stn.* **31**: 1-71.

Tadhani M B and Subhash R 2006. In Vitro Antimicrobial Activity of Stevia *rebaudiana* Bertoni Leaves. *Trop. J. Phar. Res.* 5 (1): 557-560

Tadhani M B, Jadeja R P and Rena S 2006. Micropropagation of *Stevia rebaudiana* Bertoni using multiple shoot culture. *J. cell Tiss. Res.*, 6: 545-548.

Takayama S and Akita M 1994. The types of bioreactors used for shoots and embryos. *Plant Cell Tiss. Organ Cul.* 39: 147-56.

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.

Tan S and Ueki H 1994. Method for extracting and separating sweet substances of *Stevia rebaudiana* Bertoni (English abstr.). *Jap. Patent* 06-007108.

Tanaka O 1982. Steviol-glycosides: new natural sweeteners. *Trends Anal Chem.* 1: 246-248

Tanaka O 1997. Improvement of taste of natural sweeteners. *Pure Appl. Chem.* **69**:675-683

Taware A S, Mukadam D S, Chavan A M and Taware S D. 2010. Comparative studies of *in vitro* and *in vivo* grown plants and callus of *Stevia rebaudiana* (Bertoni). *Int. J. Integra. Biol.*, 9(1): 10-15.

Thorpe T A 1993. Physiology and Biochemistry of shoot Bud Formation in vitro. In: *Advances in Developmental Biology and Biotechnology of Higher Plants.* Soh W Y, Liu J R and Komamine A (Eds.). The Korean Society of Plant Tissue Culture, Korea, pp: 210-224.

Uddin M S, Chowdhury M S H, Khan M M H, Uddin M B, Ahmed R and Baten M A 2006. *In vitro* propagation of *Stevia rebaudiana* Bert. in Bangladesh. *African J. Biotech.*, 5: 1238-1240.

Uneshi H, Ise R and Kobayashi T 1977. Purification of a stevia sweetening agent (English abstr.). *Jap. Patent* 54-030199.

Valio I F M and Rocha R F 1966. Effect of photoperiod and growth regulators on growth and flowering of *Stevia rebaudiana* Bertoni. *Jap. J. Crop Sci.* **46**: 243-248.

Van Calsteren M R, Bussière Y and Bissonnette M C 1993. Spectroscopic characterization of two sweet glycosides from *Stevia rebaudiana*. *Spectroscopy* **11**: 143-156.

Van Hooren D L and Lester H R 1992. Stevia drying in small scale bulk tobacco kilns. In: Methods to utilize tobacco kilns for curing, drying and storage of alternate crops, final report. Ontario Ministry of Agriculture and Food, Delhi.

Vanek T, Nepovim A and Valicek P 2001. Determination of Stevioside in plant material and fruit teas. *J. Food Compos. Anal.*, **14**: 383-388.

Vieira R F and Skorupa L A 1993. Brazilian medicinal plants gene bank. *Acta Hort.* **330**: 51–58.

Vis E and Fletcher H G 1956. Stevioside. IV. Evidence that stevioside is a sophoroside. *J. Amer. Chem. Soc.* **78**: 4709-4710.

Wakhlu A K and Bajwa P S 1986. Regeneration of uniform plants from somatic embryos of *Papaver somniferum* (opium poppy). *Phytomorphology* **36**: 101–105.

Wen K C 2000. The turnover rate of marker constituents in Chinese herbal medicine. *J. Food Drug Anal.* **8**: 270–277.

Withers L A 1987. Long-term preservation of plant cells, tissues and organs. *Oxford Survey Plant Mol. Cell. Biol.* **4**: 221.

Wood H B, Allerton R, Diehl H W and Fletch H G 1955. Stevioside, I. The structure of the glucose moieties. *J. Org. Chem.* **20**: 875-883.

Xiang Z P 1983. Stevia (partial English translation). General Bureau of State Farms, Heilongjiang, China.

Yamasaki K, Kohda H, Kobayashi T, Kasai R and Tanaka O 1976. Structures of stevia diterpene-glycosides: application of ^{13}C NMR. *Tetrahedron Lett.* 1005-1008.

Yang Y W and Chang C W 1979. *In vitro* plant regeneration from leaf explants of *Stevia rebaudiana* Bertoni. *Z. Pflanzenphysiol.* 93: 337-343.

Yang, Y-W, Hsing Y-I and Chang W-C 1981. Colonal propagation of *Stevia rebaudiana* Bertoni through axillary shoot proliferation *in vitro. Bot. Bull. Academia Sinica,* 22: 57-62.

Yao Y, Ban M, and Brandle J. 1999. A genetic linkage map for Stevia rebaudiana. *Genome,* 42: 657–661

Zaidan L B P, Dietrich S M C and Felippe G M 1980. Effect of photoperiod on flowering and stevioside content in plants of *Stevia rebaudiana* Bertoni. *Jap. J. Crop Sci.* 49: 569-574.

Ziegler F E and Kloek J A 1977. The stereocontrolled photoaddition of allene to cyclopent-1-ene-1-carboxaldehydes. A total synthesis of (±)-steviol methyl ester and isosteviol methyl ester. *Tetrahedron* 33: 373-380.