EFFECTS OF DIFFERENT EXPLANTS AND MEDIA ON IN VITRO REGENERATION OF CALLA LILY (*Zantedeschia albomaculata* Spreng.)

BY

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DEDICATION

I dedicate this thesis to my late father, James Kimaiyo Kures.

To him I am eternally grateful.

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ABSTRACT

There are difficulties in obtaining disease-free planting materials of calla lily, because continuously subdivided tubers accumulate pathogens. Five experiments were conducted at Egerton University, Kenya to help solve the problem. The objectives of the research were to determine effects of explants and media on in vitro regeneration of 'Black Magic' calla lily. The factors were tested in completely randomized design. In experiment I, leaf, tuber and shoot bud explants were cultured on Murashige and Skoog (MS) (1962) basal salts, supplemented with 8 g/l agar, 30 g/l sucrose, 0, 1, 2 and 4 mg/l BAP or Kinetin. The highest number of shoots per explant (2.5) and the longest shoots (3.7 cm) during the first subculture were obtained in shoot bud explants on the medium with 2 mg/l BAP. No response was observed in both leaf and tuber explants. The highest number of shoots after three subcultures was 34 for 2 mg/l BAP, compared to 3.5 for 4 mg/l Kinetin. Thus, the medium containing 2 mg/l BAP and 30 g/l sucrose proved to be the best for multiplication of shoot bud explants. In experiment II, media containing 15, 20, 30, or 45 g/l sucrose plus 2 mg/l BAP and 8 g/l agar were investigated. Tuber and leaf explants did not regenerate shoots on any of these media. The highest number of shoots (5.8) per shoot explant in the initial culture was obtained with 20 g/l sucrose, whereas there was no significant difference in shoot length after four weeks in culture. No significant difference in regenerating shoots was observed after three subcultures on media supplemented with 15, 20 or 30 g/l sucrose. Based on these results, 20 g/l sucrose plus 2 mg/l BAP proved to

be the best for rapid multiplication of shoot explants. In experiment III, media containing quarter, half or full strength MS (1962) salts plus 2 mg/l BAP were investigated. There was no significant difference in the number of shoots per explant, but the medium containing half strength MS (1962) salts produced the highest (2.3), compared to 1.9 in both the quarter and full strength MS (1962) salts. Tallest shoots (4.2 cm) were obtained in the medium supplemented with half strength MS (1962) salts. Thus, the medium with half strength MS (1962) salts proved to be the best for 'Black Magic' calla lily shoot growth and multiplication. In experiment IV, media combinations containing 8 g/l agar, 15, 20 and 30 g/l sucrose, 0, 1 and 2 mg/l IBA, or 0, 1 and 2 mg/l NAA were investigated during root initiation. Roots in IBA and the control were thin, long and branched with root hairs, compared to thick, short and without root hairs in NAA media. The highest rooting (84%) after one week was obtained with 20 g/l sucrose plus 1 mg/l IBA. A greater percentage (84%) of shoots formed roots within one week when rooted on 20 g/l sucrose + 1 mg/l IBA, compared to 70% within two weeks on 30 g/l sucrose + 1 mg/l NAA. The best overall rooting (100% within 3 weeks) was achieved on a hormone-free medium, supplemented with 15 or 30 g/l sucrose. During acclimatization, in experiment V, no significant difference occurred in the survival percentages, which were 55%, 63% and 69% for shoots rooted in the control, IBA and NAA media, respectively.

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LIST OF ABBREVIATIONS

BA	Benzyladenine
BAP	6-Benzylaminopurine
2, 4-D	2, 4-Dichlorophenoxyacetic acid
DKW	Driver Kuniyuki Walnut medium
2-iP	N^6-(2-isopentyl)adenine
IBA	Indole-3-butyric acid
HCl	Hydrochloric acid
$HgCl_2$	Mercuric chloride
MS (1962)	Murashige and Skoog (1962) medium
NAA	1-Napthalene acetic acid
NaOH	Sodium hydroxide
NaOCl	Sodium hypochlorite
RAPDs	Randomly Amplified Polymorphic DNAs
RFLPs	Restriction Fragment Length Polymorphic DNAs
STN	Shoot-tip necrosis
TDZ	Thidiazuron

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CHAPTER ONE

INTRODUCTION

Calla lilies (*Zantedeschia* species) and their hybrids can be grown as commercial cutflowers in temperate and sub-tropical climates. Forcing programmes are under development to produce calla lily cut flowers and pot plants year-round. In addition, the range of available flower colours, foliage maculation and plant heights has facilitated their use as bedding plants. *Zantedeschia* belongs to the Araceae family. While other genera of the Araceae family are endemic to S. America, Asia and Africa, the genus *Zantedeschia* is confined to the African continent. It is the most prevalent in S. Africa, but it also extends into Zimbabwe, Malawi, Zambia, Angola and Nigeria (Funnell, 1993).

Horticulturally, two important groups have been recognized. The first is typified by *Z. aethiopica,* whose foliage does not die during the winter in the native habitat. It flowers from late winter to late spring with male and female flowers interspersed in the flower spardix. The fruits turn orange and become soft and mucilaginous as they mature (Funnell, 1993). The second group contains the five remaining species *(Z. rehmannii, Z. jucunda, Z. elliottiana, Z. albomaculata* and *Z. pentlandii*) that exhibit complete foliage senescence in winter and flower during summer months. The female and male flowers are not interspersed on the spardix and the fruits remain firm and green as they mature (Funnell, 1993).

Z. aethiopica plants grow to 1.2 M height and possess ovate-cordate or hastate shaped leaves of up to 0.6 M in length (Funnell, 1993). The other species are smaller in stature, their height does not exceed 0.8 M. *Z. rehmannii* does not

exceed 0.6 M height. Leaf shape of the latter species is similar to that of *Z. aethiopica*. Leaves of *Z. rehmannii* plants are lanceolate.

The storage organ of *Z. aethiopica* is a rhizome, whereas group 2 species possess what is best known as a compact stem. The storage organs of *Zantedeschia* can be described as rhizomes, corms, or tubers (Funnell, 1993). In an effort to avoid confusion, the term rhizome is used to refer to the storage organs of group 1 species, while the term tuber is used for those in group 2.

The inflorescence comprises a spardix carrying the true male and female flowers, subtended by a coloured bract known as the sparthe. The inflorescence and sparthe are typically presented at or above foliage height on a fleshy peduncle. The sparthe may vary in shape and colour depending on the species.

Commercially, multiplication is achieved using seed, offsets/divisions and tissue culture. Only four selections are commercially produced from seed. Annual multiplication through offsets/divisions of tubers can increase the total number of flowering-sized tubers. Tissue culture techniques have been developed to provide large-scale bulking of shoots, especially of virus-free lines (Cohen, 1981). Multiplication may also be achieved by scooping dominant buds from tubers (Clark et al., 1987). Selections of *Z. aethiopica* naturally produce between 25 to 30 rhizomatous offsets after growing for six months (Welsh et al., 1988).

Generally, Aroids are propagated vegetatively ex vitro, but aseptic tissue culture techniques provide great potential in several areas of Aroid crop improvement. During incubation, tissue growth and morphogenesis in culture can

be influenced by several factors such as light, temperature, medium components, growth regulators, and stock plant conditions.

1.1 Statement of the Problem

Zantedeschia is commercially propagated from offsets/divisions, but this conventional method is too slow. There has been a problem in obtaining clean starting materials, because tubers continuously subdivided may accumulate pathogens (Manager Agriflora (Kenya) Limited, Personal communication, 2001). Cohen (1981) has reported tissue culture of calla lily hybrids, but responses and plant regeneration from leaf and tuber explants is not given. Tissue culture techniques are not currently applied for clonal multiplication of *Zantedeschia* in Kenya, forcing growers to import clean starting materials for bulking. Importation increases costs of production for *Zantedeschia.* This research aims at developing tissue culture protocols for producing calla lily locally, and hence solving the above-mentioned problems; thus slow conventional multiplication, disease accumulation, inadequate supply, and high costs of importation.

1.2 Research Justification

The horticultural industry contributes substantially to the Kenyan economy, being the third leading foreign exchange earner after tea and tourism. The cut flower industry constitutes a significant percentage of commercial horticulture, with roses being the most widely grown followed by carnations. *Zantedeschia* is improving both in volume and value on the export market.

Tissue culture techniques are now established in many research laboratories throughout the world and are being used in numerous areas of plant science. A wide range of herbaceous perennial species can be efficiently and commercially propagated using plant tissue culture techniques. Maximum productivity is achieved by finding the optimal physical and chemical conditions for multiplication, rooting and transfer to the soil. The end result has been increased quantity and quality of healthy, vigorous plants. Thus, testing various explants and media will develop a protocol for propagating calla lilies locally.

The characteristics of the tissue culture revolution can be identified as mass propagation of elite material, its improvement in the phytopathological sense, its conservation in stable, healthy form and development of new, improved genotypes. In vitro propagation can produce very large quantities of plants in a small space and independent of weather. This propensity enables rapid bulking of materials for large-scale production. With regard to calla lily, optimized tissue culture procedures, unlike conventional techniques currently employed, will ensure rapid multiplication of disease-free plants and provision of elite starting plant materials for bulking year-round and in a small space. Thus, successful micropropagation of calla lily will further enhance the Kenyan cut flower industry through local supply of planting materials to growers, reduced costs of production when materials are not imported, and increased foreign exchange earnings.

Successful application of plant tissue culture techniques in rapid micropropagation of calla lily will contribute and add to the application of plant

biotechnology in the improvement of horticultural crops in Kenya. It will also find practical use in the Kenyan calla lily cut flower industry, which is constrained by the shortage of elite planting materials. Efficient plant regeneration through tissue culture could pave way for genetic manipulation of calla lily, rapid multiplication of newly bred calla lily cultivars locally, and safe cross border exchange of germplasm, because sterile cultures pose no risk of transferring diseases.

1.3 Hypotheses

a) Explants of *Zantedeschia* 'Black Magic' exhibit varying responses and plant regeneration rates during in vitro culture due to their origin.

b) Hormones and other medium ingredients affect responses and rates of regeneration of *Zantedeschia* 'Black Magic' explants in culture differently.

c) There is no difference in acclimatization of in vitro-rooted plantlets.

1.4 Objectives

The general objective of this study was to determine the best explant and establish a micropropagation protocol for 'Black Magic' calla lily that may be adopted for rapid clonal multiplication. The specific objectives were:

a) To investigate the response of explants to sucrose, hormones (6-benzylaminopurine, Kinetin) and MS (1962) basal salts in vitro.

b) To study the effect of sucrose, indole-3-butyric acid and 1-Napthalene acetic acid on the in vitro rooting of shoots and ex vitro acclimatization of plantlets.

CHAPTER TWO

LITERATURE REVIEW

2.1 Principles of Plant Tissue Culture

The term "plant tissue culture" is used to denote the in vitro culture of a wide range of excised plant parts. It is widely employed for rapid clonal multiplication of plants, genetic engineering, germplasm preservation, storage and distribution, disease elimination and other scientific investigations. These applications are nowadays summarised in the broader term biotechnology (Hartmann et al., 1997).

The application of plant tissue for propagation may have been considered as early as 1902 when Haberlandt attempted to regenerate plants from single cells. He was not successful, but his work inspired other researchers to attempt in vitro culture of plant tissues with the first success being achieved when White (1934) reported the establishment of an actively growing clone of tomato roots. Since then plant tissue culture has advanced rapidly, becoming an integral part of the present day discipline of biotechnology.

According to Hartmann et al. (1997), plant tissue culture is based on three basic principles, namely cell totipotency, hormonal regulation of growth and development, and morphogenesis or organogenesis. The possibility of cloning isolated single cells in vitro demonstrated the fact that somatic cells, under appropriate conditions, could differentiate to form whole organisms. Minocha and Minocha (1995) described totipotency as the potential of single cells or tissues to grow and develop into multicellular or multi-organed organisms. This potential

lies in cellular differentiation, and indicates that all genes responsible for differentiation are present within individual cells. Most of these genes remain inactive in differentiated tissues or organs, but are able to express under adequate physical and chemical culture conditions when the tissues or cells are isolated. Isolated cells from differentiated tissues are generally non-dividing and quiescent (Minocha and Minocha, 1995). To express totipotency these differentiated cells must first undergo dedifferentiation and then start the redifferentiation process.

The expression of totipotency in cells and tissues of various plant species has been widely investigated through the process of somatic embryogenesis. Minocha and Minocha (1995) described this process as the best demonstration of totipotency in plant cells. Hughes (1981) suggested that totipotency was more closely associated with the retention of embryogenic competency in cells rather than organogenic competency. Thus cells that are able to undergo somatic embryogenesis are considered to be more totipotent than those that would undergo organogenesis. Cells, which have retained the capacity for a particular kind of cellular differentiation or morphogenesis, or have acquired it in response to an appropriate stimulus, are said to be competent (Hartmann et al., 1997). Individual cells or groups of cells are said to be determined when they have become committed to follow a particular genetically programmed developmental pathway and can continue towards that outcome without further influence of growth regulators (Hartmann et al., 1997).

Morphogenesis or organogenesis refers to the creation of new form and organization, where it was previously lacking; tissues or organs, which have the capacity for morphogenesis are said to be morphogenic or organogenic (George, 1993). The three forms of organogenesis in vitro include shoot organogenesis, root organogenesis and somatic embryogenesis. Morphogenesis in vitro may occur either directly from the differentiated cells of a newly explanted plant tissue without the proliferation of undifferentiated tissue or indirectly through callus cultures. Direct organogenesis often occurs when relatively large pieces of an explant are cultured and the organogenic potential of such explants is mostly influenced by their point of origin in the source plant and to a greater extent the genotype of the source plant (George, 1993).

2.2 Factors Considered in Plant Tissue Culture

Growth and morphogenesis of tissues in vitro is influenced by several factors and the key to success is through knowledge of plant regeneration and factors involved. The major factors involved are genotype, explant, culture medium and culture environment.

2.2.1 Genotype

In plant tissue culture, it is now well known that no two genotypes give similar responses under a given set of culture conditions (Nehra et al., 1990). George (1993) reported that the growth of cultured tissues of organs and

morphogenesis in vitro, are more influenced by genotype than by any other factor. Hakkaart and Versluijs (1988) reported that survival of *Alstroemeria* meristems depended on cultivar and different cultivars required varying levels of IBA in the culture medium. Although studies indicate that diverse cultivars have similar growth regulator optimum for shoot proliferation (Welander, 1985), most other reports point to the contrary (Kallack and Koiveer, 1990). The morphogenic response of pea meristems has also been shown to be genotype-specific (Kallack and Koiveer, 1990). However, the genotype-dependent behaviour of pea meristems was also strongly influenced by the exogenous growth regulator content in the culture medium.

Many genotype-dependent effects are caused by interactions between the plant's genotype and the culture environment. The behaviour of plant tissues in vitro in processes such as callus formation, growth and morphogenesis often seem to be under an over-riding genetic control, with other factors exerting only a minor effect. In some instances the observed responses have been related to identifiable genetic factors such as nuclear genes (Szakacs et al., 1988), cytoplasmic genes (Narasimhulu et al., 1988) and gene interactions (Wan et al., 1988). In *Medicago*, more plants were produced from somatic embryos in highly regenerative cultivars than from those with low capacity for regeneration, regardless of the medium employed (Brown and Atanassov, 1985). Ploidy level is also reported to have an effect in some genera. Fish and Jones (1988) found that

dihaploid lines of *Solanum tuberosum* are more likely to produce adventitious shoots from callus than are their tetraploid parents.

The ability of closely related plants to produce somatic embryos directly on explants is also under genetic control and differences between varieties are often found. Stamp and Meredith (1988) obtained somatic embryos on zygotic embryos of four cultivars of *Vitis vinifera*, but could not induce them to form on 'Pinot Noir'. The direct formation of somatic embryos on apple leaf segments depends on genotype (Welander, 1988).

In shoot cultures, whether axillary shoots can be induced to grow and proliferate and the rate of shoot multiplication on any given medium depends on genotype. For instance, the number of transferable shoots produced in 22 days of shoot culture of *Anthirrhinum* varied between 2.1 in the variety with the slowest rate of multiplication among the nine tested, and 5.9 in the variety with the highest rate (Newbury, 1986). DeWald et al. (1988) also found that on a single medium with the same growth regulator, the number of pineapple plantlets produced in 13 months from a single bud ranged from 210 to 380 for Prolera, 300 to 350 for PR-1-67 and 40 to 85 for Smooth Cayene.

Genotypic effects influence the regulation of adventitious organ formation from callus tissues. Differences in the morphogenic capacity of callus from different species or even varieties of plants have also been reported. The effect of genotype is also seen in differences in the texture, colour (Greshoff and Doy, 1972) and morphogenic capacity of callus from closely related varieties. The

results obtained in any particular test depend on the medium, the growth regulators used, and other environmental factors (Greshoff and Doy, 1972). Despite the very broad differences between species and genera, the capacity for direct morphogenesis on any given medium can also vary between closely related plant varieties. Goto (1981) found that mutant lines of the weed *Arabidopsis thaliana* had a lower shoot and root-forming capacity than the normal (parent) line, but morphogenic ability was fully restored in their hybrids. Because of genotypic specificity, media and culture environments often need to be varied from one genus or species of a plant to another.

2.2.2 Stock plant and explant factors

Growth and morphogenesis in culture are governed by properties of explanted tissues, which are additional to the immediate effects of their genetic identity. They include the manner in which stock plants have been treated and the physical and physiological condition of the explant used.

Virtually any plant part can serve as inoculum for organized development (Thorpe, 1982). These explants can give rise to organs or embryos, directly or via callus. Bhalla and Sweeney (1999) reported direct plant regeneration from leaf, stem and root explants of *Scaevola aemula,* while regenerations from embryos and cotyledons have been reported in *Anarthia scabra*, *Lepidosperma angustatum* and *Evandra aristata* (Sieler et al., 1993). Regeneration from seed, shoot tip, nodal and axillary bud explants has also been reported in the in vitro culture of

some woody plants, including *Solanum aviculare*, *Grevillea scapigera* and *Alloxylon flammeum* (Donovan et al., 1999).

The results obtained in vitro can be influenced by the way that stock plants are treated, as well as the environment in which they are grown. It is generally recognized that the best explant material is derived from healthy vigorous plants, which have been maintained in active growth without stress.

The nutritional status of stock plants may influence the growth and morphogenesis of explants. Read et al. (1983) concluded that nutrition had little effect on in vitro culture of tomato, but found that a high level of nitrogen inhibited direct rooting of cuttings in vivo. Read and Economou (1987) showed that supplying tomato stock plants with either very low or very high rates of inorganic nitrogen reduced the number of shoots that regenerated from cultured leaf segments.

The growth phase of stock plants has been shown to affect establishment of tissue cultures. In many woody plants, it is only juvenile explants, which can be established in culture. Shoot-tip explants of apple cultivars Redspur and Goldspur could be established more readily if they were taken from newly grafted shoots, rather than directly from orchard trees (Jones et al., 1985). Nodal explants obtained directly from 10-year-old *Fragaria fragrans* trees did not grow in culture, but similar explants taken from rooted cuttings of a 10-year-old tree established without difficulty (Lee and Rao, 1986). Establishment is normally improved if stock plants are given a rejuvenating treatment such as rooting or

grafting (Lee and Rao, 1986), etiolation (Ballester et al., 1989), high temperature and growth regulator treatments (Debergh and Maene, 1985).

Growth phase of stock plants also affects growth rate, shoot proliferation and organogenesis in vitro. The relative maturity of the explant nearly always has a major effect on the growth and morphogenesis of plant tissues and organs in culture. The time or season at which explants are sampled also affects results obtained in tissue culture. Changes in temperature, day length, light quality and water stress throughout the year result in mother plants having altered levels of stored carbohydrates, proteins and growth substances within their tissues (George, 1993). Chesick et al. (1990) found that bud explants of mature trees of *Larix occidentalis* gathered in June produced multiple axillary buds more rapidly than those taken at other times of the year and a higher proportion of the explants responded during in vitro culture. As conditions are not the same from one year to the next, explants taken from field-grown plants of any one genotype might have to be treated in different ways in each successive year to obtain the same result.

Choice of the correct explant is important when propagation is to be based on direct or indirect shoot initiation. Although in some species explants from many organs are capable of producing adventitious shoots, it is usually found that tissues within an organ vary in morphogenic capacity. For instance, in *Brassica carinata*, 86% of cotyledon explants, 74% of hypocotyl segments, and 26% of root segments regenerated adventitious shoots (Jaiswal et al., 1987). Explants of different origin also react to growth regulators in distinct ways, particularly in the

formation of adventitious shoots. The proximal halves of the leaf lamina of *Citrus mitis* developed adventitious buds in 3½ weeks when cultured on 2 mg/L BAP, but the distal halves of the same leaves only regenerated shoots after at least 10 weeks in culture (Sim et al., 1989).

The size and age of the explant influences the success of shoot regeneration (George, 1993). It is widely acknowledged that very small explants do not survive well in culture and that large explants may be difficult to decontaminate effectively (George, 1993). There is usually an optimum size of explants used to initiate tissue cultures. Lazzeri and Dunwell (1986) showed that 7-mm discs of *Brassica oleracea* var. *italica* leaves produced 10 times more shoots per disc than 4 mm ones. In *Hyacinthus*, the number of bulblets per bulb scale explant increased as explant length was enlarged from 1 cm to 3 cm, although the number of bulblets per unit length of explant decreased (Paek, 1982).

In general, young tissues have a higher degree of morphogenic competence than older tissues. Takayama and Misawa (1982) reported that most segments derived from young leaves of Begonia produced buds and roots, whereas those derived from mature leaves usually died. Fasolo et al. (1988) also reported that leaves taken from micropropagated shoots of *Malus* x *domestica*, formed adventitious shoots; the proportion of explants on which morphogenesis was observed, and the number of new shoots per explant was greatest in the 3 or 4 leaves nearest the tip of each shoot.

2.2.3. Temperature

Although temperature controls the overall plant development in vivo and in vitro, this factor is not usually thoroughly examined. Generally, cultures are kept at a constant temperature between 20°C and 30°C. However, the optimum temperature for growth and differentiation for a particular species should be determined, because species have different optima. *Dicentra spectabilis* shoot cultures proliferate most rapidly at 22°C, whereas explant growth and shoot proliferation are both poor at 27°C (Stimart, 1986).

The rate at which plant materials grow in vitro usually declines slowly as temperature is reduced below the optimum, while above the optimum, the rate of growth declines more rapidly and cultures frequently appear unhealthy. Rahman and Blake (1988) showed that although shoot cultures of *Artocarpus heterophyllus* (Jackfruit) produced more axillary shoots at 25°C or 30°C than at 20°C, hardly any were obtained at 35°C. They concluded that at the higher temperature respiration exceeded photosynthesis. In peach shoot-tip cultures, temperature of 21°C to 24°C ensured 80% to 90% survival, but only 7% of cultured shoots survived at 28°C (Hammerschlag, 1982).

Temperature preferences can vary between quite closely related genotypes. The best overall results were obtained at 18°C, but certain 'thermonegative' *Rosa hybrida* gave the best results at 12°C, whereas in 'thermopositive' ones, shoot proliferation was optimal at 18°C or 24°C (Horn et al., 1988). Single node cultures of *Syringa vulgaris* grew satisfactorily at 21°C in a

16-hour irradiance of $5Wm^{-2}$, but if the cultures were kept at 25°C or 27°C growth was abnormal, as indicated by leaf yellowing and curling (Pierik et al., 1988).

Organogenesis frequently reaches an optimum within a narrow range of temperature, which varies among species. In some plants the temperature for the best induction of adventitious organs is higher than that required for growth. In *Pinus radiata* adventitious buds formed most readily on juvenile explants when they were cultured in a 28°C day, and 24°C night regime, whereas the resulting shoots grew best at 24°C/20°C day/night temperature (Williams, 1996).

The temperatures at which stock plants or cultures have been maintained previously often influence morphogenesis. Kato and Ozawa (1979) reported that *Heloniopsis* shoot initiation was enhanced if explants were maintained at 16°C for 7 to 21 days before culturing at a higher temperature.

2.2.4 Light

The three characteristics of light, which most clearly influence in vitro growth and morphogenesis, are wavelength, flux density and duration of exposure (photoperiod). Their influence can be direct, on tissue already growing in vitro, or indirect on stock plants (George, 1993). In the latter case, the growth or morphogenesis displayed in vitro is modified by the light treatments applied to the stock plant before explants are excised.

The photoperiod under which cultures are exposed affects in vitro responses of plants. While the majority of plant species are normally cultured

under a 16-hour photoperiod (Taji and Williams, 1996), investigations suggest that variations exist with each species and variety in the optimum photoperiod under which it performs best in vitro. Yae et al. (1987) reported that apple shoot cultures required 16 hours of 60-$\mu mol \cdot m^{-2} \cdot s^{-1}$ for maximum rate of shoot proliferation, while *Camelia japonica* shoot cultures multiplied rapidly under both 12 and 18 hours of light (Samartin, 1989). Micropropagation under the shorter photoperiod can save lighting costs.

The wavelengths to which cultures are exposed profoundly influence growth and development. Using sources emitting light over narrow bandwidths, it has been shown that near ultra violet and blue light control the rate of growth and morphogenesis of some callus cultures. While maximum growth often occurs in darkness, calli are often moved to light when indirect morphogenesis is desired. Seibert et al. (1975) demonstrated that the combined effect of both wavelength and flux density could be important for callus growth and morphogenesis. The researchers showed that although the tissue grew in the dark, its growth was stimulated, and shoot formation was promoted to the greatest extent by near-UV light (371 nm) at the very low irradiance of 0.24 Wm^{-2}, but was inhibited when the incident flux density was more than 1.5 Wm^{-2}. However, blue light of 420 or 467 nm wavelengths stimulated maximum growth at 3 and 6 Wm^{-2}, respectively.

Irradiance is another factor influencing in vitro cultures. Light intensity influences the total light energy received by plant cultures and hence determines their growth. Most in vitro cultures are grown under light intensities, which are

about 10 to 15-fold less than those provided by natural sunlight in vivo due to the fact that in vitro cultures are non-autotrophic, and hence do not require photosynthetically active radiation (PAR).

Plants of many genera (such as Aster, Chrysanthemum and Caryophyllaceae) require relatively high irradiances for optimum shoot multiplication; culture under low light causes shoots to become succulent and hyperhydric. *Hosta* shoot cultures require high light to initiate proliferation, and tolerate both high and low irradiance thereafter (Stimart, 1986).

Murashige (1974) suggested 300 to 10,000 lux for stage III to achieve maximum rates of survival of shoots or plantlets in an exterior environment. Many laboratories find that 40 to 75 $\mu mol \cdot m^{-2} \cdot s^{-1}$ is adequate. High light levels of 155 to 200 $\mu mol \cdot m^{-2} \cdot s^{-1}$ can be beneficial for special purposes such as germinating seedlings from which explants are to be excised or germinating somatic embryos (Lazzeri and Dunwell, 1986).

2.2.5 Gases

Gases are the other factors affecting the performance of plant cultures in vitro. The gas phase above cultures include ethylene, oxygen, carbon dioxide and acetaldehyde (Hughes, 1981). Cultured tissues produce ethylene, a gaseous phytohormone, which has different morphogenetic effects such as stimulating, or inhibiting organized development, depending on the system and the nature of closures used on culture vessels (Kumar et al., 1987).

Although low flow of gases into and out of cultured plant tissues is caused by fluctuations in the temperature and atmospheric pressure of the growth room, most of the exchange of oxygen and other gases is due to diffusion (Ziv, 1991). The culture vessels are usually covered with screw caps, aluminium foil, or plastic film, which restrict gaseous exchange to different extents. The shape of the culture vessel also influences gaseous diffusion (Ziv, 1991).

Plant growth and development is dependent on respiration. Kozai (1991) suggested that the partial pressure of oxygen should be 0.5 atmospheres in the gas phase to maintain the normal rate of respiration in the cultured plant shoots. It has been shown that respiration of some plant tissues may increase as oxygen level is increased above the normal atmospheric concentration. Van der Plas and Wagner (1986) showed that the growth of potato callus is doubled if the tissue is moved from air to an atmosphere containing 70% oxygen. Adequate gaseous exchange is essential to ensure a fast rate of multiplication in most tissue cultures, but a satisfactory rate of growth can be obtained when the dissolved oxygen level is below the aerobic saturation point. Preil et al. (1988) showed that the best cell doubling time in suspension cultures of *Euphorbia pulcherima* was achieved when the partial pressure of oxygen was 60%, whereas the rate of growth declined rapidly if the oxygen partial pressure dropped below 10%.

Relative humidity is an important component of the gaseous environment that has not received much attention. This is because the RH of the culture environment is usually close to 100%. The RH of the gaseous mixture above the

medium within a culture vessel depends on its temperature and that of the medium. Where the temperature of the air equals that of the medium, and the vessel is effectively sealed, the relative humidity can range from 98% to 99.5% (George, 1993). However, the relative humidity inside vessels varies, depending on the closure material used (Short et al., 1987).

Cultures grown in high humidities are liable to become hyperhydric, but a reduction of humidity to levels below 75% to 80% may be disadvantageous for many kinds of tissue cultures. The rate of shoot proliferation of shoot cultures is poor when the relative humidity is below 75%, and cauliflower plantlets exposed to 30% and 15% RH in vitro experienced high mortality (Wardle et al., 1983). However in woody plants, highly saturated environments (99% RH) cause shoot-tip necrosis and are also implicated in increased incidences of hyperhydricity in shoot culture (Schloupf et al., 1995; Ghoraishi and Mantel, 1998).

2.2.6 Sucrose

Starting in vitro culture of any species requires basic experimentation to standardize the nutritional, growth regulator and environmental requirements of plants at each stage of the culture process (Williams and Taji, 1989; Williams, 1996). The success in plant tissue culture is largely determined by the quality of the nutrient medium, which must be suitable both in chemical composition and physical quality for culture growth during each stage of development.

In the culture of cells, tissues or organs, it is necessary to incorporate a carbon source into the medium. Sucrose is almost universally used for micropropagation purposes, because it is generally utilizable by cultured tissues. Typically all media contain sucrose in the range of 1% to 3% as a carbon source. However, there are other cases where alternative energy sources have been used for some species. Multiplication of *Almus crispa, A. cordata* and *A. rubra* shoot cultures was best on glucose, while that of *A. glutinosa* was best on sucrose (Barghchi, 1988). Direct shoot formation from *Capsicum annum* leaf discs in a 16-hour day required the presence of glucose (Phillips and Hubstenberger, 1985). Galactose has proved toxic to most plant tissues, and although it may not be able to contribute to carbon nutrition of cultured tissues, it has been found to reduce or overcome hyperhydricity in shoot cultures. Fructose has also been reported to be effective in preventing hyperhydricity (Rugini et al., 1987).

The optimum concentration of sucrose to induce morphogenesis or growth differs among genotypes, sometimes even among those that are closely related. Molnar (1988) showed that the optimum level of sucrose may depend upon the other amendments added to a culture medium. The respiration rate of cultured plant tissues rises as the concentration of added sucrose or glucose is increased. In wheat callus, it was found to reach a maximum when 90 g/L was added to the medium, although 20 g/L produced the highest rate of growth and number of adventitious shoots (Galiba and Erdei, 1986). The uptake of inorganic ions can be dependent on sugar concentration and the benefit of adding increasing quantities

of nutrients in a medium may not be apparent unless the amount of sugars is concurrently increased (Gamborg et al., 1974).

Sucrose is necessary for direct and indirect morphogenesis. It mainly acts as a carbon-energy source, but part of its effect can sometimes be due to the provision of a medium with suitable water potential. Solutions of different concentrations partly exert their effect on growth and morphogenesis through their varying osmotic potential. Lapena et al. (1988) estimated that three quarters of the sucrose necessary to promote the optimum rate of direct adventitious shoot formation from *Digitalis obscura* hypocotyls supplied energy, while the surplus regulated morphogenesis osmotically. To obtain optimum morphogenesis, it is important to vary the sucrose content of the medium. An osmotic component of sugar source was demonstrated by Murkherjee et al. (1991), who found that adding 22 mM mannitol to MS (1962) medium, containing 22 mM sucrose, considerably increased the number of shoots regenerated from seedling leaf pieces of *Solanum melongena.* Mannitol was not stimulative if there was less than 22 mM sucrose in the medium. Although the best production of adventitious shoots and corms from sections of gladiolus corms occurred on MS (1962) medium containing 6% to 9% sucrose, replacing all or part of the sucrose with mannitol did not have the same beneficial effect (DeBruyn and Ferreira, 1992).

Root formation is an energy-demanding process and carbohydrates must be provided through photosynthesis or from exogenously supplied sugars. The presence of sugar has indeed been found essential for in vitro rooting of many

species, although the optimum endogenous concentration is easily exceeded, especially when cultured shoots are capable of photosynthesizing. Some species of plants form adventitious roots and shoots during the course of stage II, but in most cases separate procedures have to be adopted, using special medium formulations and environmental controls to achieve adequate root formation.

Media of low osmotic potential are usually employed for the induction and growth of roots on micropropagated shoots. There are reports that an excessive sugar concentration can inhibit root formation. Most species root satisfactorily with the addition of 20 to 30 g/L sucrose to a rooting medium. For example, *Dendranthema* chrysanthemum shoots propagated on MS (1962) medium rooted best with 20 g/L sucrose, although higher concentrations had proved optimal for shoot multiplication (Roest and Bokelmann, 1975). 'Mini-crowns' of *Asparagus* rooted best when 60 g/L sucrose was added to MS (1962) medium (Conner and Falloon, 1990), while the best rooting of *Gentiana kurroo* (propagated on MS (1962) medium with 30 g/L sucrose), also occurred when the concentration of sucrose was increased to 60 g/L (Sharma et al., 1993). Root formation on avocado microcuttings on 0.3-strength MS (1962) salts was satisfactory with 1.5%, 3%, or 6% sucrose, and only reduced when 9% sucrose was added (Pliego-Alfaro, 1988).

Carbon source has been shown to be important in storage organ formation in some bulbous plants. Increasing sucrose concentration of MS (1962) medium from 3% to 6% improved bulb formation on cultured *Tulipa* shoots, but media containing 3% sucrose and 3% mannitol, killed explants and shoots (Taeb and

Alderson, 1990). DeBruyn and Ferreira (1992) found that adding 60 g/L to 90 g/L sucrose to MS (1962) medium and maintaining cultures at 15°C enhanced corm formation in Gladiolus. Kathryn and Anderson (1989) also reported that Dutch iris required 6% to 8% sucrose in the medium for bulbing, which also caused an increase in bulblet size and weight.

2.2.7 Inorganic salts

No other factor has received as much attention as the culture medium and as a result, numerous formulations have been published. Formulations designed by Murashige and Skoog (MS) (1962) and revised by Linsmaier and Skoog (LS) (1965), Gamborg et al. (1968), and Schenk and Hildebrandt (1972) can be regarded as standard. However, the most widely used medium is that of Murashige and Skoog (1962), which contains all the elements that are essential for plant growth. This medium is classified as a high-salt medium, compared to other formulations due to its high level of nitrogen, potassium, boron and manganese, which promote growth of most herbaceous plants, but needs modification to promote growth of other plants, especially woody species. In certain cultures, MS (1962) medium is used at half-strength (Offord et al., 1992).

A small group of basal media has been found to satisfy the mineral requirements of most plant species and tissues. Most tissue culture studies have been devoted to the definition of suitable formulations for use in culture of new

plant species. The first tissue culture media used the salt recipes developed for hydroponics, with an additional source of carbohydrates (Cohen, 1995).

The concentration of ingredients in tissue culture basal medium depends on the type of plant being cultured and the stage of development. Media with relatively high concentration of salts such as the MS (1962) or LS (1965) are most commonly employed to micropropagate herbaceous plants and several genera of woody plants. For some plants, it can be advantageous to increase or decrease the level of MS (1962) salts, while keeping the ratio of inorganic ions constant, but in other cases, improved results are obtained by varying the concentration of individual ions. The inorganic salts in a nutrient medium contribute most to the final osmotic potential, because of their greater concentration.

High levels of ammonium and nitrate have generally been preferred for callus initiation and culture, while media with lower concentrations of nitrate and ammonium ions such as Lloyd and McCown (1981) woody plant medium (WPM) have been used successfully to induce adventitious shoot formation in woody plants. Shoot and root development may gain from lower concentrations of mineral salts, and hence the use of half-strength MS (1962) or Gamborg et al. (1968) B5 media for plant regeneration is not uncommon. However, high ammonium-nitrogen concentrations in media have been associated with increased incidences of hyperhydricity in shoot cultures, as are low concentrations of gelling agents (Brand, 1993). Low calcium and boron levels in culture media have been

related to increased shoot-tip necrosis, and low levels of gelling agents exacerbate the problem (Singha et al., 1990; Abousalim and Mantel, 1994).

In callus culture, Torellor and Symington (1984) initiated callus from mature caryopses of *Lolium perenne* and also maintained the callus on a medium containing MS (1962) salts, but subcultured it to a medium with only half B5 salts to regenerate green shoots. Purnhauser et al. (1987) also found that silver nitrate dramatically improved shoot regeneration from wheat callus. Purnhauser and Gyulai (1993) showed that supplementing the stage II regeneration medium with 0.17 mg/L silver nitrate increased the number of wheat and triticale calluses, which produced shoots, and also the average number of shoots produced per callus. In *Brassica oleracea*, addition of 1 mg/L silver nitrate to the medium promoted long-term culture of callus. Without it the callus died shortly after removal from the hypocotyl explants. Shoot regeneration from the callus was also enhanced by the presence of silver ions (Williams et al., 1990).

In the culture of bulbous species, half-strength inorganics were commonly used in bulbing media for *Allium* (Hussey, 1978), but use of full-strength inorganics was necessary for optimum bulbing of *Lilium* (Takayama and Misawa, 1983). Quarter and half-strength MS (1962) inorganics were most effective in increasing iris bulblets, whereas bulblet weight was greatest at half to full-strength inorganic salt concentrations (Kathryn and Anderson, 1989).

2.2.8 Plant growth regulators

Plant growth substances or plant hormones are defined as a group of naturally occurring organic substances, which influence physiological processes within the plant, but at low concentrations (Davies, 1995). Salisbury and Ross (1992) have made a distinction between plant hormones and growth regulators by suggesting that hormones are synthesized within the plant body and may be transported to various parts where they exert their effects, while growth regulators are normally exogenously applied compounds, which mimic the functions of plant hormones. In tissue culture, the two terms are used interchangeably to mean any chemical added to the culture media, with the aim of inducing specific growth responses in explants. The processes influenced by plant hormones consist mainly of growth, differentiation and development.

Several classes of plant growth regulators have been included in tissue culture media to control morphogenesis (Thorpe, 1980). Auxins and cytokinins are the most widely used plant growth regulators in tissue culture (Taji et al., 1995), although gibberellins are also commonly used. The ratio between auxins and cytokinins may determine the type of the resulting morphogenesis. A large number of plant species respond to a suitable auxin/cytokinin balance by forming shoots and roots. Auxins include the naturally occurring indole-3-acetic acid (IAA) and the synthetic auxins indole-3-butyric acid (IBA), 1-naphthalene acetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D). In tissue culture systems, auxins have been employed in callus cultures and root initiation

(Krikorian, 1995). Generally, treatment with auxins considerably speeds the process of rooting and produces more roots. During induction of somatic embryogenesis from immature cotyledons of *Glycine max*, Lazzeri et al. (1988) discovered a highly significant interaction between the concentration of auxins and sucrose in the medium. The number of embryos obtained decrease if a high concentration of either auxins or sucrose was combined with a low concentration of the other. In addition, a high frequency of somatic embryo formation resulted under 1% to 2% sucrose and 6.25 to 25 mg/L NAA, or 4% sucrose and 50 mg/L NAA. Ranch et al. (1986) previously used 5 mg/L 2,4-D plus 6% sucrose to obtain maximum embryogenesis in *Glycine max*, while Lippmann and Lippmann (1984) found 1 mg/L 2,4-D plus 1% sucrose satisfactory.

Despite the occurrence of endogenous cytokinins in whole plants, many tissues and small organs isolated in vitro are unable to sufficiently synthesize these substances to sustain growth. Naturally occurring cytokinins include Zeatin, N-(2-isopentyl)adenine (2-iP) and dihydrozeatin. Several synthetic cytokinins and cytokinin-like compounds have been developed and are now much more widely used in tissue culture than the naturally occurring compounds. Examples of such compounds include Kinetin, 6-benzylaminopurine (BAP), and Thidiazuron.

Cytokinins have been shown to be responsible for stimulating cell division, when applied exogenously either alone or in the presence of auxins (Krikorian, 1995). Cytokinins are also added to promote adventitious shoot formation and axillary shoot proliferation in tissue cultures (George, 1993).

However, the effectiveness of cytokinins in promoting shoot proliferation in newly initiated cultures, particularly those of woody perennials, can depend on the time of the year and the phenology of the shoots from which explants are excised (Brand, 1993). The interaction and balance between the growth regulators supplied in the medium, and the growth substances produced endogenously regulate growth and morphogenesis in vitro.

Over 80 different gibberellins have been identified, but only gibberellic acid (GA_3) has most frequently been used in plant tissue culture media. When gibberellic acid (GA_3) is added to culture media, it sometimes produces effects of similar nature to those of auxins. High concentration of GA_3 (greater than 1 to 8 mg/L) induces growth of undifferentiated callus cells and occasionally promotes the growth of callus, when in combination with auxins and low levels of cytokinins (Gautam et al., 1983).

In some plants, gibberellic acid alone can induce adventitious shoot formation and also act as a substitute for auxins in the induction of shoot formation (Sekioka and Tanaka, 1981); precise gibberellin/cytokinin ratio (instead of auxin/cytokinin ratio) may then be required. Gibberellins may also increase the number of organs formed. Shoot regeneration from *Rosa hybrida* callus could be induced using 1 to 5 mg/L BAP depending on the cultivar, but adding 0.3 mg/L GA_3 to the medium increased the number of shoots produced (Valles and Boxus, 1987). Gibberellic acid has also been added to tissue culture media to improve shoot elongation before rooting of in vitro shoots (Valles and Boxus, 1987).

Rhizogenesis usually follows treatment with auxins alone, or with mixtures containing more auxins than cytokinins. High levels of cytokinins (0.5 to 10 mg/L) generally inhibit or delay root formation, and also prevent root growth and the promotive effects of auxins on root initiation (Hartmann et al., 1997). Cytokinins are, therefore, usually omitted from shoot culture media at stage III, when shoots are to be rooted to generate plantlets. However, few reports show that cytokinins can sometimes induce or promote root growth or adventitious root formation in the absence of auxins (Nemeth, 1979). In nearly all cases, only low rates of cytokinins have been effective. Boxus and Terzi (1988) advocated for the addition of 0.5 mg/L Kinetin and auxins to rooting medium for strawberry and several woody plants, after finding that this Kinetin concentration had a bacteriostatic effect and did not impair rooting.

2.3 Common Problems Associated with Tissue Culture

2.3.1 Shoot tip necrosis (STN)

Shoot tip necrosis describes a physiological disorder found in most tissue culture conditions. It is characterized by browning of plant shoot tips, which finally leads to their death or that of the entire cultured shoot. Tip necrosis has been especially observed in shoot cultures and is sometimes associated with hyperhydricity. Several hypotheses have been put forward to explain the cause of shoot tip necrosis, but deficiency of cations such as boron (Maso and Guttridge, 1974) and calcium (McCown and Sellmer, 1987) have been reported as the most likely causes. Kataeva et al. (1991) found that many shoots of *Malus* x *domestica*

and *Camellia sinensis* developed apical necrosis, browning and dropping leaves if after 3 subcultures on a medium containing cytokinins, they were transferred to a cytokinin-free medium for 2 to 3 passages to promote rooting. Shoot apices may also sometimes die if plants lose water rapidly during in vitro hardening. Ghashghaie et al. (1992) also showed that *Rosa hybrida* shoot apices became necrotic during this stage in vessels fitted with perforated lids, and plugged with cellulose fibre.

In some plants such as *Cercis canadensis* STN prevails after several subcultures have been accomplished (Yusnita et al., 1990), while in *Psidium guajava* it occurs in rapidly growing cultures after prolonged subculturing, if shoots are allowed to grow longer than 3 cm (Amin and Jaiswal, 1988).

According to Singha et al. (1990), calcium ion is a major cation that helps balance other anions within the plant. It is involved in the structural and physiological properties of cell membranes and the middle lamella of cell walls. Its deficiency results in poor root growth, blackening and curling of apical leaf margins followed by a cessation of growth and death of shoot tips. Shoot tip necrosis has been linked to calcium imbalance, which is caused by a high relative humidity environment in the culture vessels; the high humidity reduces evapotranspiration from the plantlets, leading to aberrant mass flow of calcium to the growing tips, which then die (Abousalim and Mantell, 1994).

During in vitro experimentation with tip-necrotic shoots, Debergh (1988) observed calcium deficiency and linked it to tip necrosis that occurred in

Amelanchier, Betula, Populus, Sequoia, Ulmus and other woody plants, although the extent of damage varied from genotype to genotype within the species investigated. In macadamia shoots, poor ventilation and high temperatures around the culture vessels and to a lesser extent the medium calcium concentration affected the occurrence of STN (Mulwa and Bhalla, 2000). An investigation into the prevention of tip necrosis was carried out by Barghchi and Anderson (1996) and Xing et al. (1997) in *Pistacia vera* and *Castaena sativa,* respectively. In both species, increasing the calcium concentration significantly reduced shoot tip necrosis. However, Mulwa and Bhalla (2000) reported that increasing calcium concentration in the medium beyond 6 mM resulted in increased tip necrosis symptoms in macadamia, especially when cultures were located in areas with inadequate air circulation. This showed that macadamia shoot tip necrosis was more related to the humidity levels in the culture vessel than with the calcium levels of the medium.

Shoot tip necrosis can often be cured by increasing the concentration of calcium in the medium or by changing the physical environment in which the cultures are grown so that transpiration is increased. Singha et al. (1990) reduced the incidence of STN in *Cydonia oblonga* by increasing calcium concentration in the MS (1962) medium and adjusting the concentration of other salts. However, the resulting medium proved unsatisfactory, because the altered concentration of nitrate and ammonium ions supported poor shoot growth.

Because calcium is not remobilised within plant tissues, actively growing shoots require a constant supply of ions from the medium through mass flow in the transpiration stream. When the atmosphere surrounding the culture vessels is such that it promotes excessive evaporation from the medium, a vapour-saturated environment is created around the shoots in vitro. This in turn retards the process of transpiration through which calcium is carried to shoot tips, thereby causing an 'artificial' deficiency, which is reflected in high incidences of STN. Singha et al. (1990) reported similar observations on shoots of quince, and Williams (1993) on shoots of *Ptilotus exaltatus*. Thus both the calcium level in the culture medium and the culture environment need to be considered when trying to address the problem of shoot tip necrosis.

2.3.2: Hyperhydricity

Hyperhydricity also referred to as glassiness, vitruosity or vitrescence is a wide spread problem in tissue culture and especially in woody plant cultures (Paques, 1991; Ziv 1991; Curtis and Shetty, 1996). The symptoms of this disorder may vary depending on the plant species, but in general affected plants appear translucent and water soaked, mostly with light-green, thick and brittle leaves (Debergh et al., 1992). Vitreous plants have poor multiplication and survival in culture and can pose serious problems in commercial applications (Paques, 1991).

The hyperhydric condition can affect all tissues and organs in vitro. It is conditioned by the manner in which a culture has been treated, and hence it is a

physiological disorder. The walls of border cells on hyperhydric leaves appeared thin and often damaged, with rounded and non-protruding pores (Ziv and Ariel, 1992). Hyperhydric leaves of *Datura insignis* possessed a high proportion of abnormal stomata, characterized by deformed guard cells (Miguens et al., 1993). In general, hyperhydric shoots exhibit reduced palisade parenchyma in leaves, general increase in stem cell volumes, accompanied by hypo-lignification in vessels and tracheids, and lack procambium strands between leaf primordia and stems on which they develop (Vieitez et al., 1985).

Hyperhydricity has been found to occur as a consequence of a number of factors such as high temperature, low light irradiance (Stimart, 1986), high relative humidity (Paques, 1991), low or high concentration of potassium, calcium chloride, calcium, cobalt (Lashem and Sachs, 1985), high cytokinin concentration (Kataeva et al., 1991), low concentration of gelling agent (Debergh et al., 1981) and media with high concentration of ammonium ions (Kevers et al., 1984; Vieitez et al., 1985). Hyperhydricity is also more likely to occur in tightly sealed test tubes than in those covered more loosely (Zimmerman et al., 1988). Kevers et al. (1984) also suggested that hyperhydricity might be induced when an initial stress (such as excess cytokinins or ammonium ions) modifies the phenol level in tissues, thereby enhancing the activity of both soluble and membrane-bound peroxidase enzymes.

Several approaches have been used to reduce the occurrence of hyperhydricity in vitro. Searching for ideal growth conditions is likely to

minimize its incidence (Rugini et al., 1987), while use of environmental conditions, which limit growth makes its occurrence more likely. Reducing the temperature and relative humidity inside culture vessels has been shown to reduce the occurrence of hyperhydricity. Taji and Williams (1989) found that the hyperhydricity of shoots regenerated from hypocotyl and cotyledon segments of *Clianthus formosus* could be reduced by keeping the cultures at 5°C for 10 days before returning them to ambient temperature. Similarly, the extent of hyperhydricity in *Olearia microdisca* node cultures was reduced when the vessels were placed under 5°C, although this temperature reduced the rate of shoot multiplication (Williams and Taji, 1991). Reduction in the level of cytokinins and ammonium in culture media, use of larger culture vessels, more frequent subculturing, increase in the level of gelling agents and use of permeable films as vessel covers have also been shown to reduce the incidences of vitrification (Paques, 1991; Speer, 1994). However, some of these measures lead to a reduction in the rates of multiplication in some plant species (Pierik, 1987).

2.3.3 Somaclonal variation

Somaclonal variation refers to phenotypic variation, either genetic or epigenetic in origin, displayed among somaclones. The causes and origins of somaclonal variation are manifold and relate to both internal and external aspects of plant tissue culture. It is caused either by the in vitro culture system itself or pre-existing physiological or genetic variation in somatic cells of plants (Swartz,

1991). Gross changes in the genome including endopolyploidy, polyteny and amplification or diminution of DNA sequences can occur during somatic differentiation in normal plant growth and development. Thus differences in both the frequency and nature of somaclonal variation may occur when regeneration is achieved from different tissue sources. Furthermore, the processes of de-differentiation and redifferentiation may involve both qualitative and quantitative changes in the genome and different DNA sequences may be amplified or deleted during these changes in the state of the cell.

The phenotypic anomalies produced by in vitro propagation systems can be heritable, partially heritable or not at all transmissible through meiosis (Sibi, 1990). This phenomenon is highly undesirable, particularly when the aim of the in vitro culture is to maintain the phenotype or genotype-specific characters of a plant (Zimmermann, 1988). Thus monitoring somaclonal variation under micropropagation regimes is crucial. However, in plant breeding, somaclonal variation may be a useful tool for development of new crop cultivars with desirable characters such as dwarfism, thornlessness, disease, salinity or water stress tolerance (Fourr'e et al., 1997; Norton and Skirvin, 1997). However, its application in plant breeding has many problems such as depression in vigour and yield, and unwanted upsets in growth, fertility and vigour.

Several factors have been found to influence the level of somaclonal variation. For example, the regeneration system used (Jain et al., 1998), culture conditions, number of subcultures (Jain et al., 1998), and some specific in vitro

procedures like protoplast isolation (Kawata and Oono, 1998), gene transfer (Wang et al., 1996), and cryopreservation (Fukai et al., 1994). Cytological studies indicate that during protoplast culture a high frequency of errors in microtubule synthesis, spindle formation, spindle orientation, chromatid segregation and cross wall formation occurs, resulting in extensive variation in chromosome number and structure. Growth regulators are also important in somaclonal variation because they cause transient phenotypic modifications, which although inherited mitotically during plant growth are not sexually transmitted, but epigenetic.

Somaclonal variation in tissue-cultured plants can be measured in a number of ways. Phenotypic variations characterized by morphological or protein markers have been used to detect somaclonal variations in tissue-cultured plants of aspen (Noh and Minocha, 1990) and beet (Sabir et al., 1992). Variations caused by genomic mutations affecting the number of chromosomes (ploidy) can be detected through cytometry, as in *Pinus* (Wyman et al., 1997), or chromosome counting, as in a number of woody species (Jehan et al., 1994). Chromosome counting, however, is time-consuming and does not detect changes in chromosome volume and DNA content.

The genetic markers that have been used in detecting somaclonal variations include isozymes, restriction fragment length polymorphic DNA (RFLPs) and simple sequence repeats (SSR) or microsatellite markers (Wang et al., 1996; Rani and Raina, 1998). Restriction fragment length polymorphic DNA

and randomly amplified polymorphic DNA (RAPDs) are the most widely applied techniques in somaclonal variation studies of woody plants.

2.3.4 Tissue blackening or browning

Damage resulting from the production of dark pigments is usually most severe during the initial stages of culture, and ceases to be a problem once explants have commenced growth. Results obtained by Garton and Moses (1986) with shoots and lateral buds of *Alnus oregona* are typical. The extent of blackening and growth inhibition, which occurs in cultures, depends on genotype, being especially severe in genera that naturally contain high levels of tannins or other polyphenols.

Tissue blackening occurs through the action of copper-containing oxidase enzymes (catechol oxidase), often called polyphenol oxidases, phenolase and tyrosinase (Lerch, 1981), which are released, synthesized or presented with suitable substrates and oxidative conditions when tissues are wounded or senescing. Juvenile tissues are often less prone to browning on excision than older ones (Muhitch and Fletcher, 1984), although very young explants of coffee more likely exhibit phenolic oxidation than older ones (Duhem et al., 1988).

Discolouration can also occur in cultured tissues after a period of growth. Some plants such as *Parthenium hysterophorus* (Wickham et al., 1980) produce callus that is especially liable to become discoloured and then necrotic after it has been cultured for sometime. Browning which results from the oxidation of simple

phenols is not necessarily detrimental to morphogenesis. In pieces of *Asplenium nidus-avis,* tissues became completely brown after 2 to 3 days in vitro, but still developed new green clusters of cells within two months (Fernandez et al., 1991).

Browning of tissues can be minimized by pre-treating plant materials in which black exudates can be a problem through washing and rinsing of explants after isolation and before transfer to culture and frequent transfers to fresh media (Sudarono and Goldy, 1991). The ability of activated charcoal (AC) to adsorb growth-inhibitory compounds prevents the build-up of polyphenols in the medium, but because AC can also adsorb growth regulators and other components of the medium, it should be used with caution. Etiolation or growing of stock plants in darkness or very low light intensity has been found to reduce tissue blackening (Marks and Simpson, 1990).

Browning is often more pronounced on one medium than another, showing that it could be caused by inappropriate nutrients. Browning has been shown to be less severe on a dilute medium than on a high-salt medium such as that of MS (1962) (Uosukainen, 1987; Samartin, 1989). It has been suggested that the high potassium level in the MS (1962) medium accentuates tissue browning of explants (Barghchi and Anderson, 1996). Of the numerous ways through which tissue blackening can be prevented in vitro, no single method is effective on all species and with some plants the problem can still be troublesome. However, it is recommended that a combination of treatments be adopted.

2.3.5 Microbial contamination

Plants are invariably infested with bacteria, fungi, viruses, yeast and pests. These organisms may be present on all external surfaces or in small crevices. They could also be concealed as systemic pathogens and microbial contaminants, which can only be detected and eliminated later in culture through elaborate procedures. The proportion of contaminated cultures depends on the type of explant selected. Enjalric et al. (1988) obtained 21% aseptic cultures from 3-mm axillary buds of *Hevea,* 42% from 6-mm shoot tips, and 74% from 1-mm shoot apices. Roots and other underground organs of soil-grown plants are not the best materials for initiating cultures, because they are invariably highly contaminated. However, the level of contamination may be less if plants, from which explants are to be taken, have been grown in containers of sterilized soil. Bulb explants of *Hyacinthus* were much more difficult to free from contaminants than explants from other organs, such as corms (Hussey, 1975).

Explants obtained from etiolated shoots tend to give rise to cultures with less contamination than normal explants. O'Riordain (1988) suggested that microorganisms on the original plant material were spread over a larger surface area during etiolation. Murasaki and Tsurushima (1988) found that etiolation eliminated internal microbial contamination of *Cyclamen persicum* petioles.

Shoots of many plants are less contaminated when they are in active growth than at other times. Survival of apical meristems of *Vitis rotundifolia* was high if they were excised from slow, but actively growing shoots (Gray and

Benton, 1991). Surface contamination of plant materials can be reduced if some thought is given to growing or sampling plants. Pre-culture treatment can be essential (Debergh and Maene, 1981). Taji et al. (1995) recommended spraying systemic pesticides and fungicides a few weeks before collection of explants. Le Roux and Van Staden (1991) sprayed Eucalyptus stock plants with benomyl (1 g/L) twice a week for 2 weeks before explants were collected.

Simple surface sterilization does not always remove microbial contamination or infection. Sterilants such as hypochlorite, which destroy superficial contaminants, are not usually effective in eliminating those hidden within plant tissues. Falkiner (1990) has recommended use of antibiotics for prevention of disease rather than for treatment of infection. However, use of antibiotics is limited by the fact that such compounds modify the growth and development of plant tissues and may be phytotoxic (Falkiner, 1990).

To achieve sterility in explants, several chemicals have been employed based on the length of explant exposure, chemical concentration and species in question. Hypochlorite solutions (both calcium and sodium) at 0.1 to 3% for 15 to 30 minutes are most commonly used after explants have been washed in water (Taji et al., 1995). Solvents such as ethanol (70% to 80%), ethyl and isopropyl alcohol (70%) have also been found effective (Cole, 1996). Some heavy-metal salt solutions such as mercuric chloride and silver nitrate (0.1% to 1%) can also be applied to sterilize explants (George, 1993; Cole, 1996). Addition of detergents is beneficial in increasing contact of chemicals and microbes on explants with

surface hair, scales or lenticels (Cole, 1996; Taji et al., 1995). Explants are normally rinsed several times with sterile distilled water to remove all traces of the sterilizing agents.

2.4 Acclimatization

Acclimatization is a critical stage of micropropagation of many species, because of poor survival after transfer to greenhouse or field conditions. The poor survival is occasioned by the wide difference in anatomical and physiological features existing between plants growing ex vitro and those growing in vitro (Sha-Valli Khan et al., 1999). Water stress and poor photosynthetic capacity are the two main factors contributing to poor survival (Preece and Sutter, 1991). In vitro-cultured plantlets are characterized by a thin cuticle (Sutter and Langhans, 1979), altered stomatal morphology (Donnelly and Vidaver, 1984) and stomatal malfunctioning (Marin et al., 1988). Acclimatization of micropropagated plantlets should, therefore, be done through a process that encourages development of a protective leaf-wax layer, proper functioning of the photosynthetic machinery and regulation of stomatal functioning (Al-Ahmad et al., 1998).

Grout (1988) reported that strawberry leaves formed in vitro on a medium containing sucrose portrayed an overall low photosynthetic activity at transplanting, or during acclimatization. They concluded that strawberry leaves formed in vitro function only as storage organs and serves as sources of nutrients for new developing leaves. Accordingly, larger leaves may improve acclimatization success by increasing the quantity of reserves. However, Yue et

al. (1993) reported that strawberry leaves formed in vitro on a medium supplemented with sucrose are able to photosynthesize under in vitro and ex vitro conditions. The leaves formed in vitro may function as sources of nutrients for ex vitro newly formed and expanding leaves, but they also play an important role in photosynthesis, thereby promoting the autotrophic growth of plantlets. More successful in vitro culture and acclimatization may be realized if the photosynthetic capacity of the leaves formed in vitro is used to its maximum potential (Yue et al., 1993).

Poor success during plantlet acclimatization has been attributed partially to weak vascular development at the root-to-shoot interface (Ziv, 1986; Pierik, 1987). Apter et al. (1993) concluded that the in vitro-formed adventitious root systems of Asian jasmine proved to be fully capable of continued expansion and growth during acclimatization. They also observed continuous root-to-shoot vascular connections in micropropagated Asian jasmine. This conclusion conflicts with the hypothesis that in vitro-developed roots cannot survive ex vitro conditions and must be replaced during acclimatization (Pierik, 1987).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Experimental Site

This study was conducted in the Biotechnology Laboratory (Department of Horticulture, Egerton University) located on Tatton Farm at Njoro, Kenya. The farm lies approximately at latitude 0° 23' South and longitude 35° 35' East at a mean altitude of 2200 m above sea level.

3.2. Plant Materials

Calla lily (*Zantedeschia albomaculata*) cultivar Black Magic materials used in this research were obtained from Agriflora (Kenya) limited (Sian-Nakuru), which is a commercial flower farm. Tubers that were freshly harvested and air-dried in an open sheltered shed for at least one week were obtained from the farm. Tubers measuring at least 30 mm diameter were selected for use, because tubers of this size can achieve 100% natural flowering. Some of the tubers collected were planted in containers in the greenhouse to be used as a source of leaf explants. Others were kept in the laboratory until buds produced small shoots that were then used as shoot apical explants. Ultimately, the different explants (tuber, shoot bud and leaf sections) were subjected to the treatments.

3.3 Explant Preparation and Culture Initiation

Initial explant sterilization involved washing the various explants separately for 30 minutes in running tap water, followed by vigorous shaking in

soapy water for 15 minutes, and thereafter immersion in 70% ethanol for two minutes. The explants were then treated with sodium hypochlorite (5% for tubers and 2% for leaves) for 10 minutes followed by four rinses in sterile distilled water. Explants were then trimmed by cutting-off damaged parts and sectioned into desired sizes. Tuber explants and sections containing the shoot bud were cut into cubes measuring approximately 5 mm x 5 mm x 5 mm, while leaf explants were sectioned into discs of 5 mm diameter.

The explants were inoculated into 125-ml flasks containing 25 ml of the various initiation media. The flasks were incubated in a culture room maintained at a temperature of 27 ± 1°C and 16 hours of light. After 8 days in culture, contamination in shoot bud explants (90%) was higher than in tuber explants (8%) and leaf explants (5%). This contamination prompted the additional use of another sterilizing agent, 0.1% mercuric chloride in subsequent experiments. The explants were stirred in the 0.1% mercuric chloride for 5 minutes after washing and treatment with ethanol and sodium hypochlorite. This treatment reduced the level of contamination in the subsequent experiments in all explant types and particularly in shoot bud explants, which initially exhibited very high percentage of contamination.

3.4 Media Preparation

Stock solutions were prepared for Murashige and Skoog (MS) (1962) macronutrients, micronutrients, vitamins, iron-EDTA and hormones. Each

medium was prepared by adding an appropriate volume of each stock solution and growth regulator into the desired volume of water. Sucrose was weighed and dissolved into each medium and the pH adjusted to 5.8, using 1 N HCl and 1 N NaOH. Thereafter, agar (0.8%) was weighed and added into each medium and melted in the microwave. About 25 ml of each medium was then dispensed into flasks or 5 ml test tubes before autoclaving at 100 kPa and 121°C for 18 minutes.

3.5 Experiments

3.5.1 Experiment 1: Effect of explants, BAP and Kinetin on shooting

Four levels each of BAP (0, 1, 2, and 4 mg/L) and Kinetin (0, 1, 2, and 4 mg/L) were tested on three types of explants (tuber, apical shoot and leaf explants) to determine the level that would best stimulate shooting and subsequent shoot multiplication. The basal medium consisted of full strength Murashige and Skoog (MS) (1962) salts. Sucrose was supplied at the rate of 30 g/L to the medium, gelled using 8 g/L agar. Sterile explants were inoculated into flasks containing 25 ml of each medium. The cultures were incubated in a culture cabinet maintained under 16 hours of light and 27 ± 1°C. Each treatment was assigned four explants of each kind, and replicated three times. The experiment was set up as a 4 x 3 factorial arrangement in a Completely Randomized Design for each type of hormone.

Observations on number of shoots per explant and shoot elongation began seven days after placing the explants in the hormone treatments. Data on number

of shoots per explant and shoot length were recorded for the first four weeks, after which subculturing was carried out and data on the cumulative number of shoots produced at the end of three subcultures were recorded for each treatment.

3.5.2 Experiment II: Effect of sucrose on shooting

In this experiment, the hormone level (2 mg/L BAP) that gave the best shooting response in experiment I was combined with varying levels of sucrose (15, 20, 30 and 45 g/L) to determine the effect of sucrose on shoot regeneration and multiplication on the three explant types. The medium contained full strength MS (1962) basal salts. Each treatment had four explants of each kind, replicated three times. The experiment was set up as a 4 x 3 factorial arrangement in a Completely Randomized Design. Explants were subcultured after recording data, at four weeks interval. Parameters that were assessed included shoot number and elongation. Observations began seven days after initiation of cultures and data was recorded as the number of shoots per explant and percentage of explants with shoots, in each treatment and replication. The total number of shoots produced per cultured explant at the end of three subcultures was recorded for each treatment.

3.5.3 Experiment III: Effect of MS (1962) basal salts on shooting

In this experiment, Murashige and Skoog (MS) media were augmented with the hormone level (2 mg/L BAP) and sucrose level (20 g/L) that gave the best response in the preceding two experiments. These were combined with either

a quarter, half or full strength MS (1962) basal salts, to determine the effect of MS (1962) salts on shooting of the three explant types. Each treatment had four explants of each kind, replicated three times. The experiment was set up as a 3 x 3 factorial arrangement in a Completely Randomized Design.

Explants were subcultured after recording data, at four weeks interval. Parameters that were assessed included shoot number and elongation. Observations began seven days after initiation of cultures and data was recorded as the number of shoots per explant and percentage of explants with shoots, in each treatment and replication. The total number of shoots produced per cultured explant at the end of three subcultures was recorded for each treatment.

3.5.4 Experiment IV: Effect of hormones and sucrose on in vitro rooting

In vitro regenerated shoots were used in this experiment with IBA (0, 1 and 2 mg/L) or NAA (0, 1 and 2 mg/L) to determine the best rooting medium for 'Black Magic' calla lily. Sucrose was added at various levels (0, 15, 20 and 30 g/L) to full strength MS (1962) basal medium. Four shoots and three replications represented each treatment. The experimental layout for each hormone was 3 x 4 factorial arrangement in a Completely Randomized Design. The IBA and NAA effects were analyzed separately. Observations of root induction were conducted on a weekly interval. Data on percentage of shoots that formed roots were recorded at one-week interval for four weeks.

3.5.5 Experiment V: Acclimatization of regenerated plantlets

To determine the survival percentage of shoots during acclimatization, 113 rooted plantlets were removed from their culture tubes and washed under running tap water to remove adhered agar. Forty, 38 and 35 rooted shoots, regardless of sucrose level, from hormone-free, IBA and NAA-augmented media, respectively, were acclimatized. The plantlets were individually potted in plastic bags containing sand and soil mix (2:1, v/v) and placed on a greenhouse bench. Each potted plantlet was covered with a clear polythene bag to maintain high humidity. The polythene covers were removed after 5 days, while watering was gradually reduced. After 28 days (4 weeks) the percentage number of shoots that survived the acclimatization process was recorded. The experiment was set up in a Completely Randomized Design.

3.6 Data Analysis

Data of the assessed variables were subjected to the analysis of variance. Where the F-test at $P = 0.05$ was significant, mean separation was performed using either the Least Significant Difference test or the Duncan's Multiple Range test, depending on the number of means that were being separated.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Effect of Explant, BAP and Kinetin on In Vitro Shoot Regeneration, Elongation and Multiplication of Calla lily

The initial responses on explants included tissue browning, microbial contamination (bacterial and fungal) and shoot elongation in shoot meristem explants. Tissue browning was observed at the bases of explants within the first week, but was not severe enough to kill or adversely affect growth of the explants. This condition did not persist after explants were subcultured. Explant contamination in shoot, leaf and tuber explants was 90%, 8% and 5%, respectively, within the first week of culture. This was considered primary contamination, which occurs during the first two weeks after culture inoculation due to surface bacteria and fungi not killed during sterilization. Secondary contamination occurs after two weeks if plants are infected by systemic contaminants (Lightbourn and Deviprasad, 1990).

Frequent contamination problems are encountered during the in vitro culture initiation stages of most root and tuber explants (Taji and Williams, 1996). Such tissues require extensive disinfection, which may be hampered by the sensitivity of tissues to high concentration of disinfecting chemicals. As a result, long periods of time elapse before the next stages in micropropagation can be undertaken (Taji and Williams, 1996). However, techniques to rid explants of contamination must be developed to suit individual species and explants. In this research, the use of an additional disinfectant, containing 0.1% mercuric chloride

($HgCl_2$) produced encouraging results. Primary contamination levels decreased to less than 10% in shoot explants and 2% in both tuber and leaf explants.

During the first week, shoot explants elongated rapidly and produced 1 to 2 axillary shoots although those in the medium without hormones (control) were stunted and regenerated no axillary shoots (Tables 1and 2). Shoot growth and elongation occurred 3 to 4 days after explant transfer to culture media. There was 100% shoot growth in shoot explants, compared to 0% in both tuber and leaf explants in all media tested, even after 8 weeks in culture. The tuber explants darkened and deteriorated in culture.

Regeneration from leaf tissues is routinely used for plant propagation in a few plant species (Evans and Sharp, 1986). In a number of plant species genetic transformation has employed inoculation of leaf discs in vitro with a strain of *Agrobacterium tumefaciens,* which harbours an engineered plasmid (Billings et al., 1988). Leaf discs of *Passiflora edulis* var. *flavicarpa* formed callus when cultured on modified MS (1962) medium supplemented with 2 mg/L NAA, 2 mg/L IAA and 0.4 mg/L Kinetin (Amugune et al., 1993). They also formed little calli and shoots when cultured on a medium supplemented with 2 mg/L BAP and 0.5 mg/L Kinetin. However, leaf discs of *P. edulis* var. *edulis* did not form callus, roots or shoots on any of these media (Amugune et al., 1993). Park et al. (1995) reported regeneration of potato shoots from leaf tissues, but responses to published regeneration regimes showed cultivar specificity (Wheeler et al., 1985).

Table 1. Effect of explant and BAP on proliferation of shoots on calla lily explants after 4 weeks in culture

BAP (mg/L)	Explant			Mean
	Shoot	Leaf	Tuber	
	Regenerating shoots (%)			
0	100[a]	0[b]	0[b]	33.3
1	100[a]	0[b]	0[b]	33.3
2	100[a]	0[b]	0[b]	33.3
4	100[a]	0[b]	0[b]	33.3
Mean[Z]	100[a]	0[b]	0[b]	
	Number of shoots per explant			
0	1[a]	0[b]	0[b]	0.3
1	2[a]	0[b]	0[b]	0.7
2	2[a]	0[b]	0[b]	0.7
4	2[a]	0[b]	0[b]	0.7
Mean	2[a]	0[b]	0[b]	
	Shoot length (cm)			
0	2[a]	0[b]	0[b]	0.7
1	3[a]	0[b]	0[b]	1.0
2	4[a]	0[b]	0[b]	1.3
4	3[a]	0[b]	0[b]	1.0
Mean	3[a]	0[b]	0[b]	

[Z]Means not followed by a letter or followed by the same letter within a letter series and within each variable are not significantly different at $P = 0.05$

Table 2. Effect of explant and Kinetin on proliferation of shoots on calla lily explants after 4 weeks in culture

Kinetin (mg/L)	Explant			Mean
	Shoot	Leaf	Tuber	
	Regenerating shoots (%)			
	100	0[b]	0[b]	33.3
1	100[a]	0[b]	0[b]	33.3
2	100[a]	0[b]	0[b]	33.3
4	100[a]	0[b]	0[b]	33.3
Mean[Z]	100[a]	0[b]	0[b]	
	Number of shoots per explant			
0	1[a]	0[b]	0[b]	0.3
1	2[a]	0[b]	0[b]	0.7
2	2[a]	0[b]	0[b]	0.7
4	2[a]	0[b]	0[b]	0.7
Mean	2[a]	0[b]	0[b]	
	Shoot length (cm)			
0	1[a]	0[b]	0[b]	0.3
1	2[a]	0[b]	0[b]	0.7
2	2[a]	0[b]	0[b]	0.7
4	2[a]	0[b]	0[b]	0.7
Mean	1.8[a]	0[b]	0[b]	

[Z]Means not followed by a letter or followed by the same letter within a letter series and within each variable are not significantly different at $P = 0.05$

Billings et al. (1988) observed no shoot growth in leaf explants of blueberry on a medium without hormones, but obtained shoot regeneration on a medium containing N^6-(2-isopentyl)adenine (2-iP) (5, 10, 15 and 20 μm) and zero or low concentration of NAA. Kunisaki (1980) regenerated plantlets of *Anthurium andreanum,* using vegetative buds (meristems) as explants, while Geier (1986), working with *Anthurium scherzerianum,* induced plantlets from leaf explants.

Efficient, repeatable and rapid in vitro regeneration systems are a prerequisite for using recent advances in biotechnology to improve crop plants (Mohammed et al., 1992). Genetic transformation is one of the interesting areas of biotechnology for plant breeders because characteristics can be added with minimal alteration of the target plant's genome. Therefore, direct shoot morphogenesis from primary tissues is more desirable than going through a callus phase (Stiekman et al., 1988). It is generally accepted that regeneration of plantlets from callus tissue frequently results in chromosome abnormalities that would be undesirable in commercial propagation (Evans and Sharp, 1986).

In the current research, *Zantedeschia* tuber explants failed to respond to both Kinetin and BAP. Nevertheless, shoot regeneration has been achieved in other species, using tuber explants. Ng' (1988) achieved direct plant regeneration from tuber discs of *Dioscorea rotundata* (White yam) on MS (1962) medium, supplemented with 0.5 or 1 mg/L BAP. Complete formation of plantlets occurred within 3 months in culture.

In this research, once tuber and leaf explants failed to respond in both BAP and Kinetin media, the experimental design was changed to a one factor Completely Randomized Design and data was subjected to analysis of variance testing the effect of hormone on shoot explants (table 3).

The initial growth rates among treatments were similar until after two weeks when a significant difference in the number of shoots was observed (Table 3). The highest number of shoots (2.2) was obtained in the medium containing 2 mg/L BAP, while the maximum length of shoots (3 cm) was obtained in the medium augmented with 1 mg/L BAP. In contrast, 1.5 shoots in 4 mg/L Kinetin and 1.4 cm in 2 mg/L Kinetin resulted. After four weeks in the initial culture, the highest number of shoots per explant (2.3) resulted on the medium supplemented with 4 mg/L BAP, compared to 1.8 with the best Kinetin (4 mg/L) concentration.

Shoots obtained on 1 mg/L BAP medium were very small, difficult to handle and not suitable for rooting. It is not necessarily desirable to obtain the largest possible number of adventitious or axillary shoots from cultures, because such shoots are not of good quality (short stems, short internodes and difficult to separate). To overcome this problem, Rugini and Verma (1983) used two media for stage II of almond shoot tip cultures. The first medium incorporated 0.7 mg/L BAP and 0.1 mg/L NAA to induce shoot multiplication. The second medium, with a lower level of BAP (0.2 mg/L) and no auxins, induced shoots to grow to a sufficient size for rooting.

Table 3. Effect of BAP and Kinetin on shoots and shoot length of calla lily

	BAP (mg/L)[Z]				Kinetin (mg/L)[Z]				
	0	1	2	4	0	1	2	4	
Time (wks)	Number of shoots per explant								LSD
1	1.0	1.5	1.6	1.7	1.4	1.2	1.3	1.4	NS
2	1.5^{bc}	2.0^{ab}	2.2^{a}	1.8^{ab}	1.2^{c}	1.3^{c}	1.3^{c}	1.5^{bc}	0.46
3	1.5	1.8	2.5	2.4	1.2	1.5	1.5	1.7	NS
4	1.5	1.8	2.2	2.3	1.3	1.7	1.7	1.8	NS
	Shoot length (cm)								
1	0.8	1.2	1.3	0.7	0.9	1.0	0.9	0.9	NS
2	1.2^{cd}	3.0^{a}	2.4^{ab}	1.9^{bc}	1.0^{d}	1.3^{cd}	1.4^{cd}	1.3^{cd}	0.90
3	1.4^{b}	3.2^{a}	3.2^{a}	2.2^{b}	1.3^{b}	1.4^{b}	1.5^{b}	1.5^{b}	0.98
4	1.5^{c}	3.4^{ab}	3.7^{a}	$2.5b^{c}$	1.4^{c}	2.0^{c}	1.7^{c}	1.7^{c}	1.13

[Z]Means not followed by a letter or followed by the same letter within a letter series along weeks are not significantly different at $P = 0.05$

Subculturing of explants after four weeks to fresh BAP medium of the same composition essentially enhanced shoot proliferation (Figure 1). Few axillary shoots developed on growth-regulator-free and Kinetin media. The number of axillary shoots increased markedly in BAP media and this difference remained constant throughout the three subcultures. Multiplication rate (shoots per explant) gradually increased during progressive transfers (Figure 1).

The increase in the number of shoots with subculturing was similar to that obtained by Clemente et al. (1991) in *Artemisia granatensis*, in which the highest shoot proliferation rate was achieved with 0.44 μM BA, but most of the axillary shoots were vitrified by the fourth subculture. Although elongation of the main shoot was greater in the absence of BA, axillary shoots increased markedly with BA treatment throughout the four subcultures (Clemente et al., 1991).

Subculture intervals vary with plant species and 4 to 8 weeks have been reported. This stage of subculturing is considered the most crucial in any clonal micropropagation programme (George, 1993; Hartmann et al., 1997). Debergh and Maene (1977) found that several axillary shoots, and later adventitious shoots, developed in *Pelargonium* if apices of individual buds on proliferating meristems were subcultured to a fresh medium. Koblitz and Koblitz (1983) also reported that seedling cotyledon explants of *Lycopersicon esculentum,* on which shoot buds had formed directly, could be induced to produce more shoots if those, which had already elongated were removed and cultured separately.

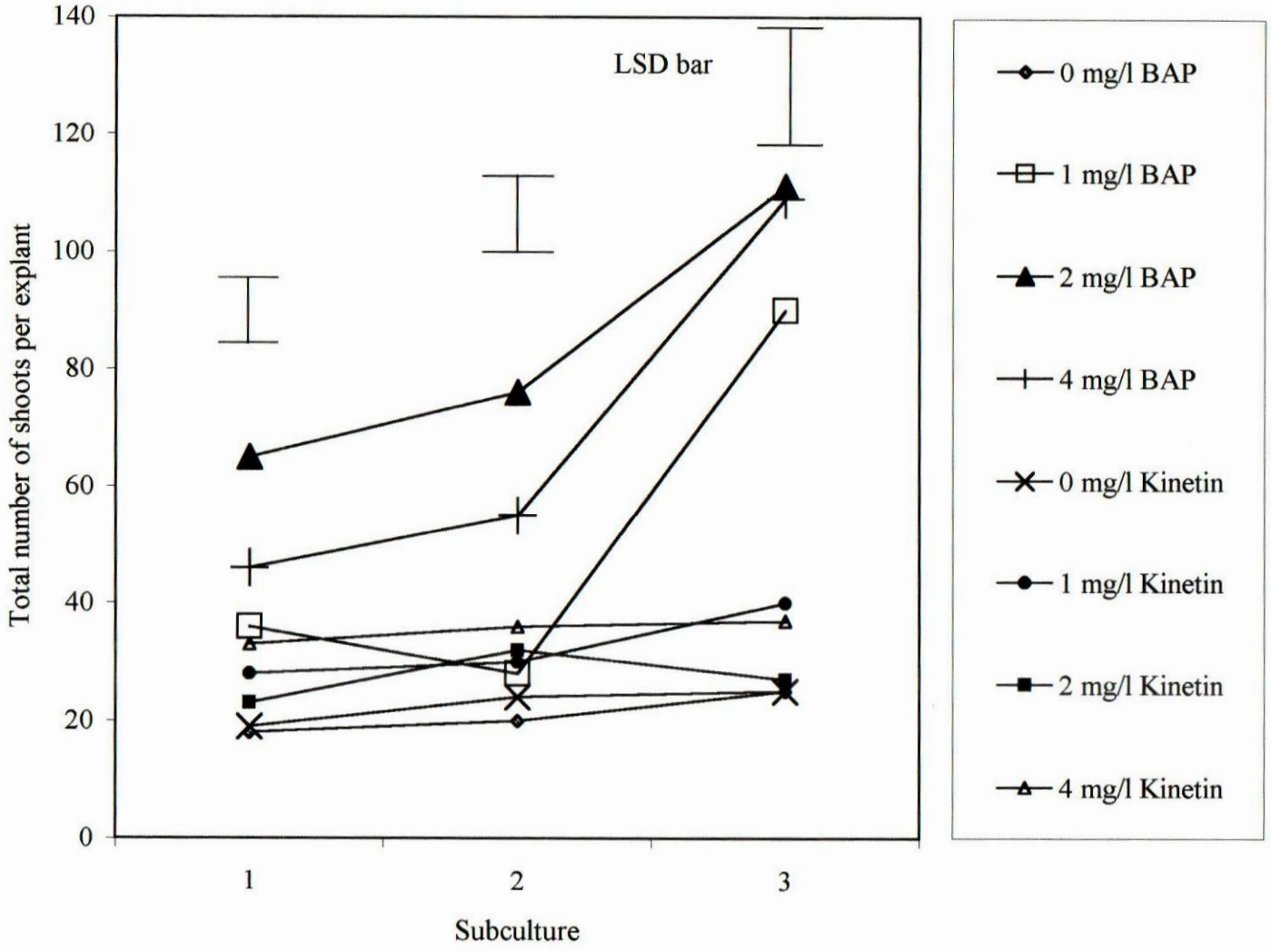


Fig. 1. Effect of hormone on number of shoots per explant during three subcultures of four weeks each

In the current study, nearly all concentrations of BAP produced more axillary shoots than the Kinetin levels tested (Figure 1). Axillary and adventitious shoot proliferation is stimulated after adding high cytokinin levels in the medium (George, 1993; Taji et al., 1995; Hartmann et al., 1997). From these results, probably higher than 4 mg/L Kinetin could be required for enhanced shoot proliferation in calla lily. In Geraldon wax (*Chamelaucium uncinatum*), a medium supplemented with 4.4 or 22.0 μM BA resulted in the highest number of explants that displayed enhanced axillary bud growth (Page and Visser, 1989). Saam et al. (1987), induced multiple shoots in shoot tip cultures of common bean (*Phaseolus vulgaris*) using BA at 0.3 to 3 mg/L. It is apparent from the current experiment that optimum adventitious shoot formation on shoot explants of calla lily is promoted by subculturing.

Due to the high levels of explant contamination, some shoots died before reaching the next subculturing stage, and hence the number of shoots subcultured at every interval was not uniform for all treatments. Therefore, data was extrapolated to make comparisons among the treatments. The number of excisable shoots (0.5 to 1.5 cm long) per explant was summed over 10 weeks and divided by the initial number of explants. The concentration of 2 mg/L BAP promoted the highest multiplication rate (34-fold), compared to 3.5-fold for 4 mg/L Kinetin, which was the best medium enriched with Kinetin (Figure 2).

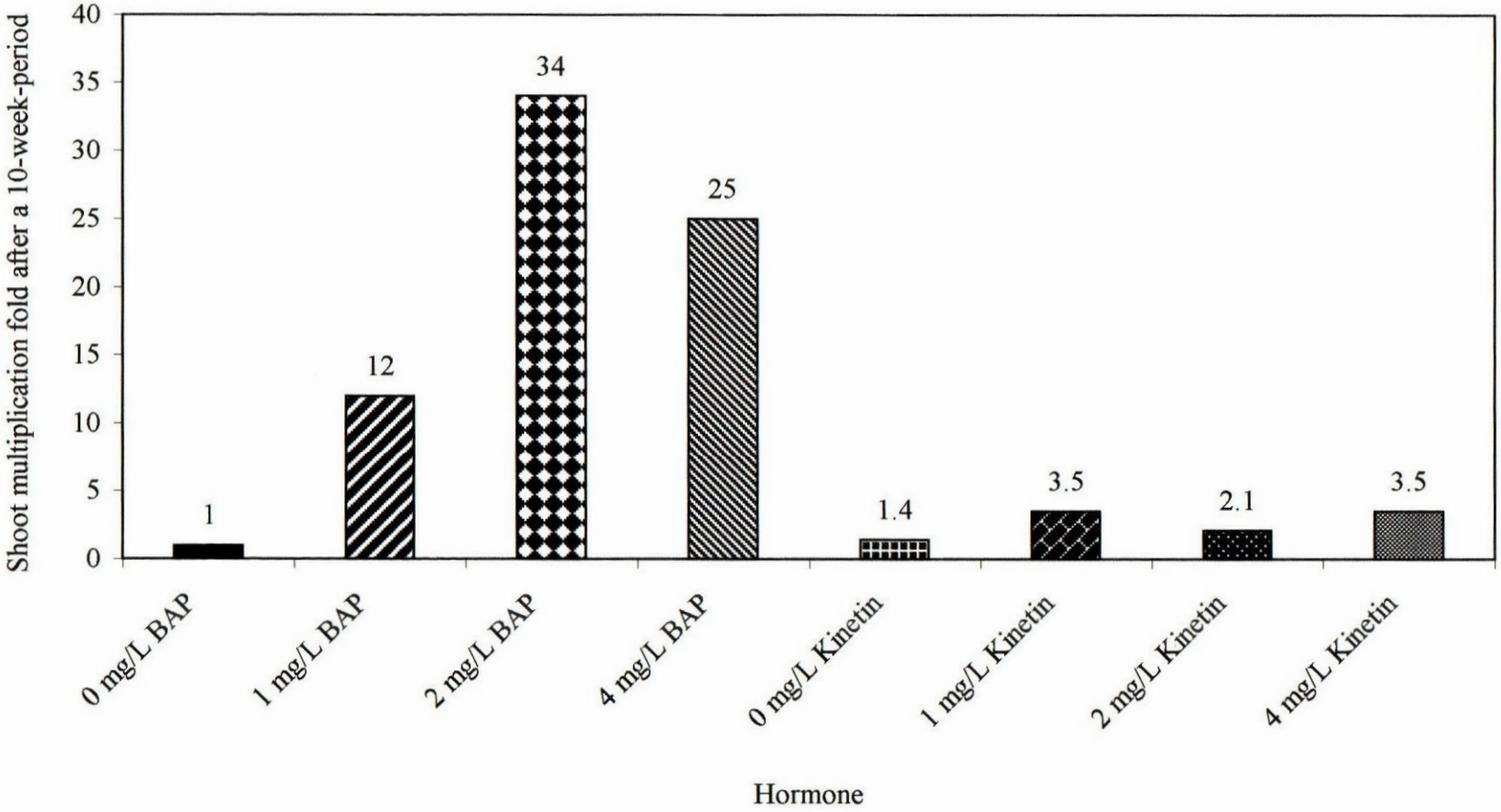


Fig. 2. Effect of hormone on shoot multiplication fold per explant of calla lily cultivar Black Magic after a 10-week-period

4.2 Effect of Sucrose on Shooting

Unlike tuber and leaf explants, shoot explants (100%) in all treatments responded by elongating rapidly within four days in culture, whereas leaf and tuber explants did not exhibit any growth response. These results are similar to those obtained in the preceding experiment, in which both tuber and leaf explants failed to respond to all concentrations of Kinetin and BAP (Tables 1 and 2).

The initial growth rates of shoot explants were similar in all treatments until after 14 days when significant differences in shoot length and number of shoots per explant were detected (Table 4). The tallest shoot (2.1 cm) by day 14 regenerated on the medium augmented with 15 g/L sucrose + 2 mg/L BAP, compared to 1.8 cm in medium augmented with 30 g/L sucrose + 2 mg/L BAP. The shortest shoots (1.6 cm) during this period developed on the medium supplemented with 45 g/L sucrose + 2 mg/L BAP. After the 2 weeks in culture, there was no further significant difference in shoot length among the treatments. This could be due to the fact that there was no further significant influence of sucrose. However, by the end of four weeks in culture, shoots on the medium containing 15 g/L sucrose + 2 mg/L BAP still were tallest (2.2 cm), compared to (1.9 cm) shoots on the medium supplemented with 30 g/L sucrose + 2 mg/L BAP. Shoots on the medium supplemented with 45 g/L sucrose remained the shortest (1.7 cm) (Table 4).

Table 4. Effects of sucrose on number of shoots and shoot length of calla lily

	Sucrose (g/L)[Z]				
	15	20	30	45	
Time (weeks)	Number of shoots per explant				
1	1.7[b]	2.5[a]	2.1[a]	1.6[b]	0.41
2	3.2[c]	4.9[a]	3.7[b]	2.8[c]	0.49
3	3.7[b]	5.7[a]	3.9[b]	2.9[c]	0.60
4	3.8[b]	5.8[a]	3.9[b]	3.0[c]	0.66
	Shoot length (cm)				LSD
1	1.7	1.6	1.4	1.5	NS
2	2.1[a]	1.7[b]	1.8[ab]	1.6[b]	0.30
3	2.1	1.8	1.8	1.7	NS
4	2.2	1.9	1.9	1.7	NS

[Z]Means not followed by a letter or followed by the same letter within rows and variables are not significantly different at $P = 0.05$.

Significant differences in the number of shoots per explant were observed among the media throughout the four weeks in culture (Table 4). The highest average number of shoots per explant (2.5 and 2.1) at day 7 resulted when explants were cultured on MS (1962) medium supplemented with 20 g/L and 30 g/L sucrose + 2 mg/L BAP, respectively. On average, only 1.6 shoots resulted on the medium augmented with 45 g/L sucrose + 2 mg/L BAP (Table 4).

At the end of 2 weeks shoots per explant increased significantly (Table 4). The highest number of shoots (4.9) developed on the medium containing 20 g/L sucrose + 2 mg/L BAP, compared to 3.7 on the medium containing 30 g/L sucrose plus 2 mg/L BAP. After four weeks an average of 5.8 shoots developed on the medium containing 20 g/L sucrose + 2 mg/L BAP, compared to 3.9 and 3.8 on media supplemented with 30 g/L and 15 g/L sucrose, respectively. The fewest shoots (2.9) regenerated on the medium augmented with 45 g/L sucrose. Thus, at the end of the four weeks, the medium with 20 g/L sucrose and 2 mg/L BAP proved to be the best for multiplication of 'Black Magic' calla lily.

Subculturing shoots every four weeks significantly increased the rate of shoot multiplication (shoots per explant), although there was no significant difference in multiplication rates among media augmented with 2 mg/L BAP plus 15, 20 or 30 g/L sucrose (Fig. 3). The medium that most of the time promoted the lowest multiplication rate per explant contained 45 g/L sucrose. Molnar (1988) showed that the optimum level of sucrose may depend on the other amendments such as hormones and solidifying agents added to a medium.

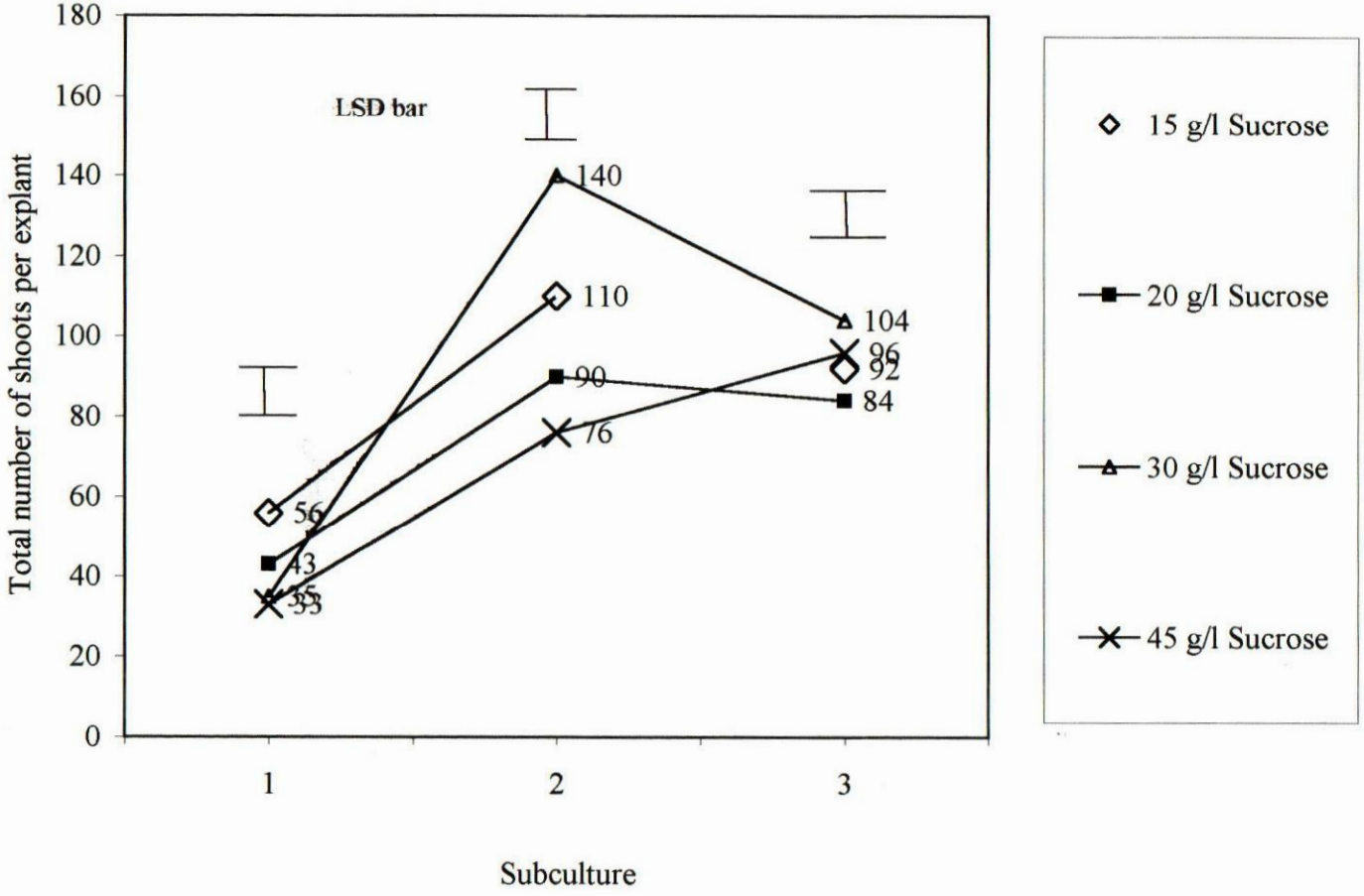


Fig. 3. Effect of sucrose on number of shoots per explant during three subcultures of four weeks each

Seingre et al. (1991) reported an interaction between sugar and gelling agent after shoot proliferation of *Malus* 'EM.9' on media gelled with six commercial products was greatest when 15 g/L sorbitol was added to the medium. With Phytogel P 8169, it was better when sucrose was added to the medium. Although sucrose had no significant effect on shoot height, the medium supplemented with 20 g/L sucrose proved to be the best for producing a greater number of shoots per explant (Seingre et al., 1991). In Chinese cabbage, established shoot cultures grew best on media containing 30 g/L sucrose. Reducing the concentration to 20 g/L halved the number of shoots formed, while high concentrations enhanced callus growth and inhibited shoot multiplication (Kee et al., 1987). Galiba and Erdei (1986) also reported that in wheat callus maximum growth resulted when 90 g/L sucrose was added to the medium, but 20 g/L produced the highest growth rate and number of adventitious shoots.

In the current research, the highest final multiplication rate (16-fold) was obtained when shoots were subcultured on a fresh medium augmented with 15 g/L sucrose + 2 mg/L BAP for three culture passages (Figure 4). This value was higher than the 11-fold and 7-fold obtained on media supplemented with 2 mg/L BA plus 30 g/L and 20 g/L sucrose, respectively. However, the lowest rate of multiplication (5-fold) was obtained on the medium supplemented with 45 g/L sucrose + 2 mg/L BAP (Figure 4). This suggests that 15 to 30 g/L sucrose is suitable for multiplication of 'Black Magic' calla lily. Above this concentration, sucrose seemed to inhibit shoot growth and the shoots developing per explant.

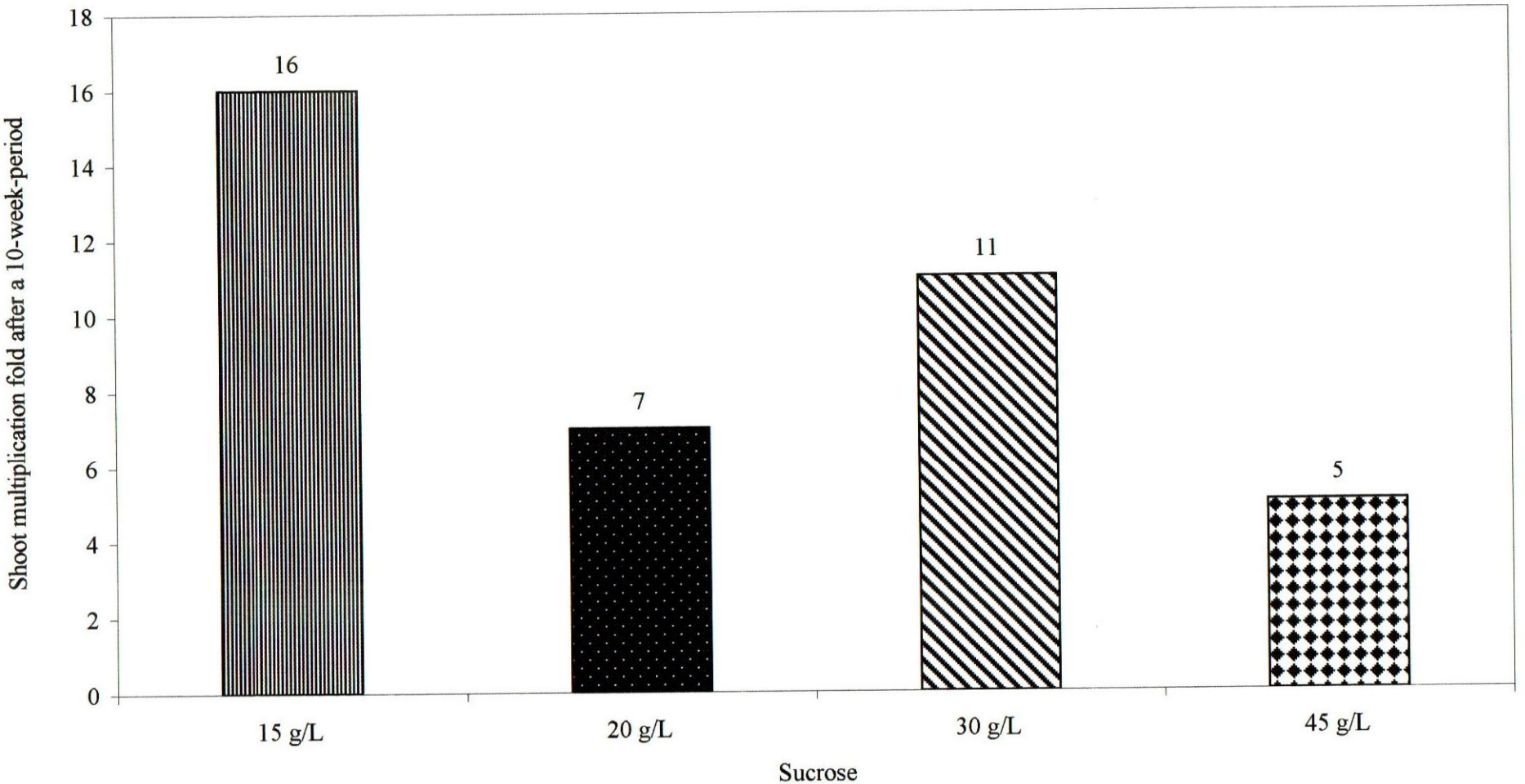


Fig. 4. Effect of sucrose on shoot multiplication fold per explant of calla lily cultivar Black Magic after a 10-week-period

4.3 Effect of MS (1962) Basal Salts on Shoot Regeneration

The initial responses of explants cultured on different levels of MS (1962) basal salts were similar to those of the preceding experiments, in which tuber and leaf explants did not exhibit any growth response. Nevertheless, shoot explants responded by growing and elongating rapidly within three to four days in culture.

There was no significant difference in the number of shoots regenerating per explant among quarter, half and full strength MS (1962) basal salts throughout the four weeks in culture (Table 5). Regardless, by the end of two weeks, the highest number of shoots (1.9) resulted on half strength MS (1962) salts, compared to 1.6 and 1.6 on quarter and full strength MS (1962) salts, respectively. The number of shoots developing per explant increased with time and by the end of four weeks, half strength MS (1962) salts still promoted the highest shoots (2.3), compared to the 1.9 shoots on quarter or full strength MS (1962) salts.

A significant difference was detected in shoot length from the first to the fourth week (Table 5). The medium supplemented with half strength MS (1962) basal salts produced the tallest shoots (4.4 cm long), while that supplemented with quarter strength MS (1962) basal salts produced the shortest shoots (2.9 cm) throughout the experimental period. The medium supplemented with full strength MS (1962) salts had an intermediate effect, which was not significantly different from that of half strength MS (1962) basal salts. At the end of four weeks, the increase in shoot length from week three was small, suggesting that shoot growth had reached a plateau in all media during this period in culture (Table 5).

Table 5. Effects of MS (1962) salts concentration on shoots per explant and shoot length during in vitro culture of calla lily

Time (weeks)	MS (1962) salts concentration [Z]			
	Quarter	Half	Full	LSD
	Number of shoots per explant			
1	1.6	1.6	1.4	NS
2	1.6	1.9	1.6	NS
3	1.8	2.4	1.7	NS
4	1.9	2.3	1.9	NS
	Shoot length (cm)			
1	1.9^{b}	2.9^{a}	2.6^{ab}	0.7
2	2.6^{b}	4.1^{a}	3.4^{a}	0.8
3	2.8^{b}	4.2^{a}	3.8^{ab}	1.1
4	2.9^{b}	4.4^{a}	4.0^{a}	1.1

[Z]Means not followed or followed by the same letter within rows and within the variables are not significantly different at $P = 0.05$

Stunted growth and even deterioration is a normal occurrence, occasioned in prolonged culture by several factors such as exhaustion of nutrients in the culture medium, accumulation of ethylene in culture vessels, and production of inhibitory by-products, among others.

Although there was no significant difference in the number of shoots developing per explant in all media strengths tested, the medium augmented with half strength MS (1962) salts proved to be the best for multiplication of 'Black Magic' calla lily shoots.

Shoot cultures of some Bromeliads showed increased survival at stage I, and improved rate of multiplication at stage II, when MS (1962) salts were added at a quarter or half strength (Mekers, 1977). In *Syringa vulgaris*, single node cultures elongated and multiplied most rapidly when grown on a medium containing full strength or 1.25-fold MS (1962) salts; however, these salt concentrations induced leaf curling and shrinkage, which were eliminated when the medium included 1.5-fold MS (1962) salts (Pierik et al., 1988).

In the current study, the medium supplemented with half strength MS (1962) salts proved to be better in terms of shoot elongation, although the difference in number of shoots among the media tested was minimal. Thus, the media supplemented with half and full strength MS (1962) salts proved optimal for 'Black Magic' calla lily shoot regeneration and multiplication.

Due to microbial contamination that killed most of the shoots, it was not possible to carry out subsequent subcultures in this experiment. Thus, only data

for the first culture passage is presented in Figure 5. Subculturing shoots after four weeks onto the same media as that used at the initiation stage, in terms of MS (1962) basal salts level, did not produce a significant effect on the number of shoots developing per explant in the first culture passage (Figure 5).

In the current study, although a maximum of 2.3 shoots developed per explant on half strength MS (1962) salts, this value might have been improved by further modifications of the medium. It has been reported that exogenous application of cytokinins in conjunction with a low concentration of auxins is more effective in shoot production than cytokinins alone (Shabde and Murashige, 1977). Smith et al. (1986) determined that multiple shoots develop when *Manihot esculentum* nodal sections are cultured on half strength MS (1962) medium, augmented with 0.05 mg/L NAA and 0.2 mg/L BAP. Sultanbawa and Phatak (1991) also demonstrated that shoots of ornamental pepper could grow on half strength MS (1962) medium, supplemented with 2 mg/L BAP, and root on the same medium, but without growth regulators. Kuo and Tsay (1977) propagated Chinese cabbage (*Brassica rapa*) through shoot tip culture of axillary bud explants on half strength MS (1962) macronutrients plus 1 mg/L NAA and 0.1 mg/L BAP. On MS (1962) medium without growth regulators each explant produced a single shoot (Kuo and Tsay, 1977). In *Allium cepa*, Falavigna and Hussey (1979) reported that 10 to 20 adventitious shoots developed per explant in response to BAP, ranging from 1 to 4 mg/L in combination with 0.5 mg/L NAA, and half strength MS (1962) salts with modified iron and organic components.

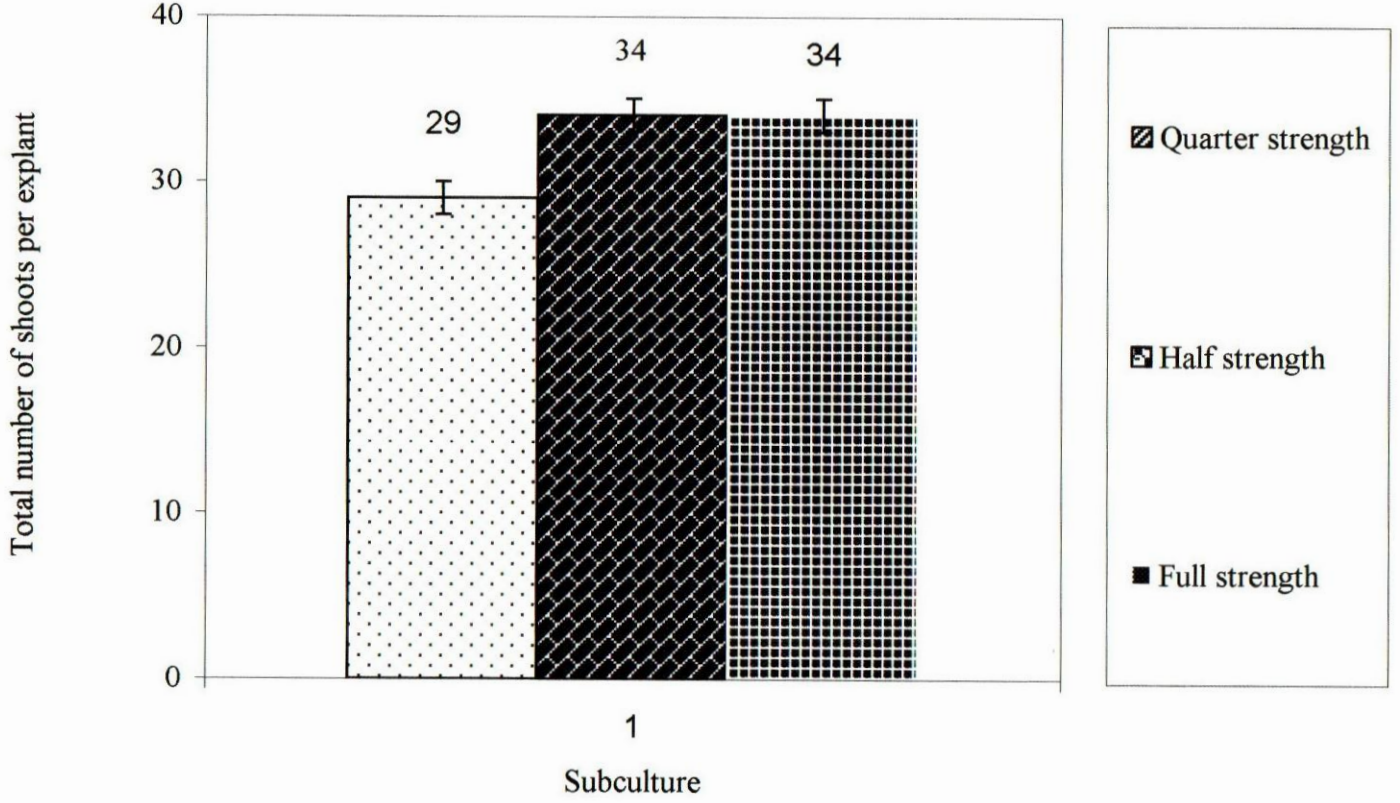


Fig. 5. Effect of MS (1962) basal salts concentration on number of shoots per explant after one subculture of four weeks. The bar indicates one S.E.

In the current experiment for 'Black Magic' calla lily, alterations of the MS (1962) salt concentration alone did not significantly affect shoot multiplication. However, there was a difference in the vigour of the shoots, which was attributed to the concentration of the MS (1962) basal salts.

Thus, basal salts interact with plant growth regulators when promoting shoot regeneration. Media components promote different growth aspects, which develop better when both the initiating and promoting compounds are all present. Auxins and cytokinins initiate cell division, whereas basal salts add nutritional value to media.

4.4 Effect of Sucrose, NAA and IBA on In Vitro Rooting

The rooting stage is the most critical step in the micropropagation of plant species if clonal mass production of material is to be possible. Both in vitro and in vivo methods have been used to induce roots in different plants with varied success rates. In the current study, significant differences among the rooting media resulted, when the effects of NAA and sucrose on rooting were analyzed weekly for four weeks (Table 6).

In the first week, no roots formed in all NAA-augmented media (Table 6). The highest rooting percentage (80%) was achieved in the medium supplemented with 15 g/L sucrose alone, compared to 69% and 67% in media containing 30 g/L and 20 g/L sucrose alone, respectively. However in the second week, there was a significant interaction between NAA and sucrose.

Table 6. Effects of sucrose and NAA on in vitro rooting (%) of calla lily shoots

Sucrose (g/L)	NAA (mg/L) 0	1	2	Mean
		Week 1		
15	80^{a}	0^{c}	0^{c}	27
20	80^{a}	0^{c}	0^{c}	22
30	69^{b}	0^{c}	0^{c}	23
Mean[Z]	72^{a}	0^{b}	0^{b}	
		Week 2		
15	93^{a}	7^{de}	8^{de}	36^{c}
20	87^{ab}	64^{c}	0^{e}	50^{b}
30	93^{a}	70^{bc}	24^{d}	62^{a}
Mean	91^{a}	47^{b}	11^{c}	
		Week 3		
15	100^{a}	40^{c}	14^{d}	51^{b}
20	87^{ab}	76^{ab}	80^{ab}	81^{a}
30	100^{a}	76^{ab}	66^{b}	81^{a}
Mean	96^{a}	64^{b}	53^{b}	
		Week 4		
15	100^{a}	65^{c}	23^{d}	63^{b}
20	100^{a}	88^{ab}	88^{ab}	92^{a}
30	100^{a}	76^{bc}	66^{c}	81^{a}
Mean	100^{a}	76^{b}	59^{c}	

[Z]Means not followed by a letter or followed by the same letter within a letter series and within a week are not significantly different at $P = 0.05$.

In the second week, 70% rooting occurred when 30 g/L sucrose was combined with 1 mg/L NAA. This value was, however, lower than the 93% rooting percentage achieved with 0 mg/L NAA plus 15 g/L or 30 g/L (Table 6). By the end of three weeks, there was no significant difference among the media supplemented with 20 or 30 g/L sucrose + 1 mg/L NAA and 20 g/L sucrose + 2 mg/L NAA. These media were not significantly different from the 0 mg/L NAA plus 15 g/L to 30 g/L sucrose media, which had achieved 87% to 100% rooting after three weeks.

After four weeks, 100% of the shoots had rooted in all media without NAA, but augmented with 15 g/L, 20 g/L or 30 g/L sucrose, whereas 88% shoots had formed roots in the medium augmented with 20 g/L sucrose + 1 or 2 mg/L NAA (Table 6). Thus, complete rooting in hormone-free media was achieved faster than in NAA-augmented media. Regenerated shoots of *Brassica oleracea* var. capitata also rooted readily on a medium containing no growth regulators (Lillo and Shahin, 1986).

Although most species root satisfactorily with the addition of 20 to 30 g/L sucrose to rooting media, there was no significant difference in percent rooting of 'Black Magic' calla lily shoots cultured on media supplemented with 15, 20, or 30 g/L sucrose alone after four weeks (Table 6). This result suggests that sucrose did not directly influence rooting of *Zantedeschia* 'Black Magic' shoots. This proved that any of the three levels of sucrose (15, 20, and 30 g/L) tested is suitable for rooting of 'Black Magic' calla lily shoots.

In spite of the interaction between sucrose and NAA, better rooting was achieved in the medium without NAA. These results are contrary to those obtained by Chee and Pool (1988) in which the percentage of rooted *Vitis* explants increased in response to increasing NAA concentration, whereas it was insufficient in the best treatment to be acceptable for micropropagation when the medium contained 3% sucrose alone. In their study, the percentage of rooted explants and explants initiating roots decreased with increasing sucrose concentration. The vigour of explanted *Vitis* shoots was highest if only 10 g/L sucrose was added to MS (1962) basal salts medium, and at this concentration they rooted well with 0.4 μM NAA. Shoot vigour declined if sucrose was above 10 g/L; erratic and inadequate levels of rooting accompanied this decline (Chee and Pool, 1988). Page and Visser (1989) have reported that a decrease of either the MS (1962) components or sucrose level increased the number of Geraldon wax (*Chamelaucium uncinatum*) shoots that rooted. A simultaneous decrease of both MS (1962) components and sucrose further enhanced root formation. However, a decrease of these constituents, but with omission of NAA from the medium reduced the number of shoots that rooted.

In Chinese cabbage roots formed in the absence of auxins but addition of auxins (IBA or NAA) increased the rooting percentage (Kee et al., 1987). Root initiation is usually markedly affected by the culture conditions during shoot multiplication just prior to rooting medium (Bressan et al., 1982). This effect could be a consequence of residual compounds in the shoot accumulated from the

multiplication medium, which can either promote or inhibit root initiation. It could also be due to the developmental and/or physiological state of the explant at that particular period of culture. Bressan et al. (1982) suggested that the BA in shoot multiplication medium is one residual compound that can affect subsequent root initiation. Shoots of common bean, measuring 1 to 2.5 cm long and obtained from cultures on basal medium without plant growth regulators or from 2 μM BA medium, formed adventitious roots 7 to 10 days after transfer to media without plant growth regulators (Mohammed et al., 1992). Passiatore and Sink (1981) also reported that 100% rooting in *Nicotiana alata* occurred within 6 to 10 days when regenerated shoots were inserted in MS (1962) medium, containing 3% sucrose and 0.8% agar, or 3% sucrose, 0.1 mg/L NAA and 0.35% agar.

Most shoots cultured in the medium supplemented with or without IBA rooted within 4 to 8 days (Table 7). The highest rooting percentage in the first week (84%) was achieved in the medium containing 20 g/L sucrose + 1 mg/L IBA, compared to 80% with 15 g/L or 20 g/L sucrose and no IBA, respectively. From the second week in culture, no significant difference in rooting percentage was observed among the media augmented with 15, 20 or 30 g/L sucrose alone and all those with IBA (Table 7). Nevertheless, the medium with 15 g/L or 30 g/L sucrose alone induced the highest (93%) rooting percentage in the second week, compared to 90% and 89% in the medium augmented with 20 g/L sucrose + 2 mg/L IBA and 20 g/L sucrose + 1 mg/L IBA, respectively (Table 7).

Table 7. Effects of sucrose and IBA on in vitro rooting (%) of calla lily shoots

Sucrose (g/L)	IBA (mg/L) 0	1	2	Mean
		Week 1		
15	80^{ab}	27^{cd}	17^{d}	41^{b}
20	80^{ab}	84^{a}	52^{bc}	72^{a}
30	69^{ab}	52^{bc}	61^{ab}	61^{a}
Mean[Z]	76^{a}	54^{b}	43^{b}	
		Week 2		
15	93	80	56	76
20	87	89	90	89
30	93	67	79	80
Mean	91	79	75	
		Week 3		
15	100	80	83	88
20	87	100	90	90
30	100	83	86	90
Mean	96	88	84	
		Week 4		
15	100	93	94	96
20	100	100	94	92
30	100	92	86	93
Mean	100	91	90	

[Z]Means followed by the same letter or no letter within a letter series and within weeks are not significantly different at $P = 0.05$

By the end of three weeks, the highest rooting percentage (100%) was achieved in rooting media augmented with 15 g/L or 30 g/L sucrose and no IBA, respectively, and that with 20 g/L sucrose + 1 mg/L IBA. The lowest rooting percentage (80%) was achieved in the medium augmented with 15 g/L sucrose + 1 mg/L IBA (Table 7). After 4 weeks in culture, most shoots on all IBA-augmented media and the control had rooted, but no significant difference in rooting percentage was observed among the media. Nevertheless, media augmented with sucrose alone and that supplied with 20 g/L sucrose + 1 mg/L IBA had achieved 100% rooting, while 94% and 93% rooting had been achieved in media with 15 or 20 g/L sucrose + 2 mg/L IBA and 15 g/L sucrose + 1 mg/L IBA, respectively. These results proved that an interaction occurred between sucrose and IBA during rooting of *Zantedeschia* 'Black Magic' shoots.

Although roots formed in the absence of auxins addition of IBA increased rooting percentage better than NAA (Tables 6 and 7). These results are similar to those obtained in Chinese cabbage (Kee et al., 1987), whereby roots formed in the absence of auxins, but addition of either IBA or NAA auxins increased the percentage rooting (Tables 6 and 7).

Visual comparison of IBA and NAA effects showed that in the first week no roots formed in all the NAA media tested, while at most 84% shoots rooted in the medium augmented with 20 g/L sucrose + 1 mg/L IBA (Tables 6 and 7). There was a difference in root morphology between IBA and NAA treatments. Roots formed in hormone-free media with 15, 20 and 30 g/L sucrose and IBA-

augmented media were long, thin and branched with root hairs, compared to short, thick and without root hairs in NAA-augmented media (Fig. 6). These results are similar to those obtained by Kee et al. (1987) with Chinese cabbage, in which roots induced by IBA or IAA were elongated, but those developing in the presence of NAA were stumpy and thick.

A greater percentage of shoots produced roots (84% versus 70%) within a shorter period of time (1 week versus 2 weeks) when placed on 20 g/L sucrose + 1 mg/L IBA versus 30 g/L sucrose + 1 mg/L NAA, respectively. This and previous findings proved that IBA is a better rooting hormone than NAA for *Zantedeschia* 'Black Magic' shoots. In *Apios americana*, 70% to 80% of the regenerated shoots rooted on solid medium, containing 0.5 or 2.5 μM IBA, while 90% of the shoots formed roots in liquid medium with 0.5 μM IBA (Wickremesinhe et al., 1990). After 6 weeks, about 50% of the shoots had formed sufficient roots for transfer to a potting mixture (Wickremesinhe et al., 1990). Furthermore, a greater percentage of the *A. americana* shoots rooted (90% versus 75%) within a shorter period of time (6 weeks versus 12 weeks) when placed on liquid versus on solid media, respectively (Wickremesinhe et al., 1990). Contrary to these results, Clemente et al. (1991) reported that incorporation of IBA, ranging from 0.49 to 14 μM, or NAA ranging from 0.53 to 16 μM into the basal medium for 3 days did not enhance either the rooting percentage (70%), or the



Figure 6. Morphology of 'Black Magic' calla lily roots developed in hormone-free (1), NAA (2), and IBA (3) supplemented media

number of roots relative to those obtained in control shoots. The presence of IAA during the entire culture period decreased the rooting percentage to 50% at 0.57 μM IAA and 0% at 17 μM IAA, whereas roots did not form with IBA or NAA at all levels tested (Clemente et al., 1991). With 'Black Magic' calla lily, the overall best rooting medium contained 15 g/L to 30 g/L sucrose and no hormones.

4.5 Acclimatization of Plantlets

During the acclimatization process, there was no significant difference in survival percentages of plantlets obtained from hormone-free, IBA and NAA-treated media (Table 8). A total of 55%, 62% and 69% plantlets from hormone-free, IBA and NAA rooting media, respectively, survived the ex vitro hardening process. These rates are quite sufficient particularly for NAA-rooted plantlets, which initially portrayed poor root morphology, compared to hormone-free and IBA-rooted plantlets (Fig. 6). These results indicated that root morphology did not significantly influence the survival rate, given that plantlets rooted in the presence of NAA developed roots that were short, thick and with few root hairs (Fig. 6). These results also proved that plantlets with longer roots such as those developing in the presence of IBA do not survive transplantation as well as does those with shorter roots. The results also revealed that 100% in vitro rooting does not necessarily translate into 100% survival ex vitro, as evidenced by the lowest survival percentage (55%) of plantlets that had exhibited the highest rooting percentage (100%) in hormone-free media.

Table 8. Survival of regenerated calla lily plantlets after acclimatization

Source of plantlets	Survival (%) [Z]
Control	55
IBA	62
NAA	69

[Z]Means not followed by a letter are not significantly different according to the F-test at $P = 0.05$

It has been reported that roots grown in vitro frequently develop no or few root hairs, which easily die after transplanting, causing loss of plantlets or cessation of growth (Preece and Sutter, 1991). Several researchers have suggested that roots developed in vitro are non-functional, and hence must be replaced with new ones when micropropagated plantlets are weaned for acclimatization in potting mixes ex vitro (Debergh and Maene, 1981; Pierik, 1987).

The presence of root hairs on tissue culture-formed roots could have significant implications for acclimatization of tissue culture-produced plant materials. Root hairs play an important role in the rhizosphere, effectively increasing the root surface area; similarly, water uptake is made more effective by the presence of root hairs (Kramer, 1983). The shortened length or absence of root hairs on tissue culture-formed roots may render them less functional to the plantlet, increasing the stress imposed during and after acclimatization.

Clemente et al. (1991) reported that 50% of *Artemisia granatensis* shoots developed roots when placed directly into covered glass jars filled with peat, for one week after which the lids were removed. After 4 to 5 weeks, plants with well-developed root systems were transferred to pots containing peat. This proved to be the best procedure for acclimatizing the plantlets (Clemente et al., 1991). In *Apios americana*, about 50% of the regenerated shoots from both sprouted tuber, stem and epicotyl explants had sufficient roots for transfer to the potting mix in 6 weeks (Wickremesinhe et al., 1990). Dipping roots cleaned-off agar in a 250-ppm Benlate solution, followed by application of the same solution to the potting mix,

increased the percentage of acclimatized plants from 10% to 30%. When shoots were rooted in liquid media and treated with Benlate, 60% of them survived (Wickremesinhe et al., 1990).

Rooted watermelon plantlets were acclimatized under the same conditions as those for tissue cultures in cell packs or tray plugs, filled with Pro Mix BX and coarse vermiculite (1:1 v/v), and covered with a clear plastic lid (Compton and Gray, 1993). After 7 days, the plants were acclimatized to ambient humidity levels by gradually removing the lid over 2 to 3 days. The plantlets were transferred to the greenhouse after 14 days and an average of 72% of the plantlets acclimatized successfully to greenhouse conditions (Compton and Gray, 1993).

In vitro-grown plants usually show rapid wilting when transferred to greenhouse or field conditions if care is not taken to maintain high humidity in their new environment. This has been attributed to the severe impairment of their water-content maintenance mechanisms (Fila et al., 1998), which result in poor regulation of leaf transpiration due to thin cuticles (Shakle et al., 1990), lack of leaf epicuticular waxes (Al-Ahmad et al., 1998), and poor stomatal regulation. Tissue culture conditions while highly promotive of shoot proliferation and plantlet growth are antagonistic to the formation of normal leaf anatomy (Ziv, 1986). Thus, careful acclimatization and planting out is an important aspect, and should not be overlooked in descriptions of in vitro culture protocols. Essential to any practical system of in vitro culture is not only a high multiplication rate of plantlet production from the explant, but also a high rate of survival during

acclimatization and in the field. Successful acclimatization is one of the primary factors limiting commercial tissue culture production systems (Hartmann et al., 1997). The widespread use of micropropagation is restricted for several reasons, one of which is the percentage of in vitro-grown plants that can be lost or damaged at the time of transfer from tissue culture vessel conditions to soil and ambient growth environments (Fila et al., 1998). The tissue culture vessel microclimate is known to play a major role in modelling the physiology and morphology of in vitro-derived plants. Zacchani et al. (1997), suggested that factors responsible for the specific effects include the elevated relative humidity and gas composition inside tissue culture vessels, temperature and radiation parameters such as light quality, intensity and photoperiod.

CHAPTER FIVE

CONCLUSIONS AND RECOMMENDATIONS

A) Conclusions

1. In the current study, *Z. albomaculata* 'Black Magic' tuber and leaf explants failed to respond to any of the media tested, whereas shoot (bud) explants responded by growing and elongating rapidly.

2. The hormones used in the study (Kinetin and BAP) showed differences in the rate of multiplication and shoot vigour. Kinetin nearly always produced fewer and less vigorous shoots, compared to the BAP levels. From the results, 2 mg/L BAP proved to be the best for shoot growth.

3. Fewer axillary shoots resulted from the growth-regulator-free medium and Kinetin-augmented medium, although *Z. albomaculata* 'Black Magic' shoot bud explants showed an absolute requirement for cytokinins to increase proliferation of axillary shoots.

4. Although no significant difference was observed in shoot length among the sucrose levels, 20 g/L sucrose in combination with 2 mg/L BAP proved to be the best in producing a greater number of shoots per explant.

5. The strength of MS (1962) salts produced no significant effect on both the shoot length and number of shoots regenerated. However, half or full

strength MS (1962) basal salts concentration proved optimal for growth and multiplication of 'Black Magic' shoots. The number of shoots and shoot vigour decreased in a quarter strength MS (1962) salts.

6. There was an interaction between auxins (IBA or NAA) and sucrose; thus, sucrose did not have a direct influence on rooting. A greater percentage of shoots (84%) produced roots within a shorter period of time (1 week) when rooted on the 20 g/L sucrose + 1 mg/L IBA medium. Nevertheless, rooting was best in growth regulator-free media, compared to IBA or NAA-augmented media.

7. Although rooting occurred in the absence of auxins, addition of IBA increased the rooting percentage better than NAA. IBA-induced roots were long, thin and branched with root hairs, compared to short, thick and without root hairs in NAA-treated media. This proved that IBA is a better rooting hormone than NAA for *Z. albomaculata* 'Black Magic' shoots.

B) Recommendations

The adoption of improved technologies is often expected to enhance production. Using tissue culture as a rapid method to create an initial stock of *Zantedeschia* cultivars would provide the foundation for the creation of healthy stocks of selected clones in a more commercially acceptable time. Thus based on

the foregoing findings, the following recommendations for adoption and further research are made:

1. Use MS (1962) basal salts at half or full strength, supplemented with 15 to 30 g/L sucrose and 2 mg/L BAP to induce high quality multiple shoots from shoot primordia explants of *Z. albomaculata* 'Black Magic'.

2. Root the resulting shoots on hormone-free media, supplemented with sucrose, ranging from 15 g/L to 30 g/L, and gelled with 8 g/ L agar.

3. Acclimatize rooted plantlets under cool humidified conditions, which can be achieved through covering each potting bag with a clear polythene bag on small-scale production, instead of the expensive mist systems.

4. Further investigate the interaction among other *Zantedeschia* cultivars, media, growth regulators, photoperiod and explants on in vitro response. This is justified by the fact that not all the explants tested responded, and only one cultivar was used in this research.

5. Investigate callus culture and in vitro tuberization, using various explants and growth regulator combinations, which might result in a positive response in terms of shoot multiplication and tuber production.

6. Formulate sterilization procedures that can effectively reduce the high contamination levels observed in the current study, particularly for tuber and leaf explants.

7. Investigate the effect of duration of exposure to root-inducing hormones on ex vitro survival of in vitro-rooted calla lily plantlets.

8. Develop ex vitro rooting protocols to eliminate stage III, which appeared to be unnecessary, because regenerated shoots rooted readily in the absence of root-inducing hormones. Elimination of stage III in vitro would reduce the cost of producing the calla lily planting materials, and hence make them inexpensive to growers.

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APPENDICES

Appendix 1. ANOVA tables for effects of hormone on shooting

Week 1

Source	DF	SS	MS	F-value	Probability
Hormone	7	0.953	0.136	1.6239	0.1993
Error	16	1.341	0.084		
Total	23	2.293			

Week 2

Source	DF	SS	MS	F-value	Probability
Hormone	7	2.651	0.379	2.9936	0.00328
Error	16	2.024	0.127		
Total	23	4.675			

Week 3

Source	DF	SS	MS	F-value	Probability
Hormone	7	4.291	0.613	2.3644	0.0730
Error	16	4.149	0259		
Total	23	8.440			

Week 4

Source	DF	SS	MS	F-value	Probability
Hormone	7	2.203	0.315	0.9509	>0.05
Error	16	5.296	0.331		
Total	23	7.500			

Appendix 2. ANOVA tables for effects of hormone on shoot length

Week 1

Source	DF	SS	MS	F-value	Probability
Hormone	7	0.847	0.121	1.3286	0.2997
Error	16	1.450	0.091		
Total	23	2.305			

Week 2

Source	DF	SS	MS	F-value	Probability
Hormone	7	10.150	1.450	5.0905	0.0034
Error	16	4.539	0.284		
Total	23	14.647			

Week 3

Source	DF	SS	MS	F-value	Probability
Hormone	7	13.635	1.947	6.4324	0.0010
Error	16	4.845	0.303		
Total	23	18.480			

Week 4

Source	DF	SS	MS	F-value	Probability
Hormone	7	15.571	2.224	5.2527	0.0029
Error	16	6.776	0.423		
Total	23	22.347			

Appendix 3. ANOVA tables for effects of hormone on subcultured shoots

Subculture 1

Source	DF	SS	MS	F-value	Probability
Hormone	7	219.167	31.310	15.0286	0.0000
Error	16	33.333	2.083		
Total	23	252.500			

Subculture 2

Source	DF	SS	MS	F-value	Probability
Hormone	7	3291.625	470.232	62.0086	0.0000
Error	16	121.333	7.583		
Total	23	3412.958			

Subculture 3

Source	DF	SS	MS	F-value	Probability
Hormone	7	74369.167	10624.167	109.6217	0.0000
Error	16	1550.667	96.917		
Total	23	75919.833			

Appendix 4. ANOVA tables for effects of sucrose on shooting

Week 1

Source	DF	SS	MS	F-value	Probability
Sucrose	3	1.538	0.513	8.0332	0.0056
Error	8	0.511	0.064		
Total	11	2.048			

Week 2

Source	DF	SS	MS	F-value	Probability
Sucrose	3	7.655	2.552	15.8499	0.0010
Error	8	1.288	0.161		
Total	11	8.943			

Week 3

Source	DF	SS	MS	F-value	Probability
Sucrose	3	12.974	4.325	20.7923	0.0004
Error	8	1.664	0.208		
Total	11	14.638			

Week 4

Source	DF	SS	MS	F-value	Probability
Sucrose	3	13.487	4.496	20.9312	0.0004
Error	8	1.718	0.215		
Total	11	15.205			

Appendix 5. ANOVA table for effects of sucrose on shoot length

Week 1

Source	DF	SS	MS	F-value	Probability
Sucrose	3	0.155	0.052	3.4747	0.0706
Error	8	0.119	0.015		
Total	11	0.273			

Week 2

Source	DF	SS	MS	F-value	Probability
Sucrose	3	0.423	0.141	6.6567	0.0145
Error	8	0.170	0.021		
Total	11	0.593			

Week 3

Source	DF	SS	MS	F-value	Probability
Sucrose	3	0.266	0.089	2.6573	0.1196
Error	8	0.267	0.033		
Total	11	0.533			

Week 4

Source	DF	SS	MS	F-value	Probability
Sucrose	3	0.363	0.121	2.7507	0.1123
Error	8	0.352	0.041		
Total	11	0.715			

Appendix 6. ANOVA table for effects of sucrose on subcultured shoots

Subculture 1

Source	DF	SS	MS	F-value	Probability
Sucrose	3	134.250	44.750	8.3906	0.0075
Error	8	1.42.667	5.333		
Total	11	10.917			

Subculture 2

Source	DF	SS	MS	F-value	Probability
Sucrose	3	978.667	326.222	7.6309	0.0099
Error	8	342.000	42.750		
Total	11	1320.667			

Subculture 3

Source	DF	SS	MS	F-value	Probability
Sucrose	3	8892.250	2964.083	16.6756	0.0080
Error	8	1422.000	177.750		
Total	11	10314.250			

Appendix 7. ANOVA tables for effects of MS (1962) basal salts on shooting

Week 1

Source	DF	SS	MS	F-value	Probability
MS salts	2	0.059	0.030	1.652	0.3736
Error	6	0.152	0.025		
Total	8	0.211			

Week 2

Source	DF	SS	MS	F-value	Probability
MS salts	2	0.219	0.109	2.1906	0.1931
Error	6	0.300	0.050		
Total	8	0.519			

Week 3

Source	DF	SS	MS	F-value	Probability
MS salts	2	0.684	0.34	3.7966	0.0860
Error	6	0.541	0.090		
Total	8	1.225			

Week 4

Source	DF	SS	MS	F-value	Probability
MS salts	2	0.247	0.123	1.2907	0.3418
Error	6	0.573	0.096		
Total	8	0.820			

Appendix 8. ANOVA tables for effects of MS (1962) salts on shoot length

Week 1

Source	DF	SS	MS	F-value	Probability
MS salts	2	1.417	0.708	10.1187	0.0120
Error	6	0.420	0.070		
Total	8	1.837			

Week 2

Source	DF	SS	MS	F-value	Probability
MS salts	2	3.493	1.746	9.7167	0.0131
Error	6	1.078	0.180		
Total	8	4.571			

Week 3

Source	DF	SS	MS	F-value	Probability
MS salts	2	3.326	1.663	9.9766	0.0124
Error	6	1.000	0.167		
Total	8	4.326			

Week 4

Source	DF	SS	MS	F-value	Probability
MS salts	2	3.900	1.950	6.17582253	0.0349
Error	6	1.896	0.316		
Total	8	5.795			

Appendix 9. ANOVA table for effects of MS (1962) salts on subcultured shoots

Source	DF	SS	MS	F-value	Probability
MS salts	2	5.556	2.778	0.7041	0.042
Error	4	12.650	3.290		
Total	8	18.049			

Appendix 10. ANOVA tables for effects of NAA and sucrose on rooting

Week 1

Source	DF	SS	MS	F-Value	Probability
NAA (A)	2	30969.745	15484.873	587.5703	0.0000
Sucrose (B)	2	102.190	51.095	1.9388	0.1728
AB	4	204.379	51.095	1.9388	0.1476
Error	18	474.373	26.354		
Total	26	31750.687			

Week 2

Source	DF	SS	MS	F-Value	Probability
NAA (A)	2	29054.547	14527.274	94.3005	0.0000
Sucrose (B)	2	3140.267	1570.134	10.1922	0.0011
AB	4	5147.392	1286.848	8.3533	0.0005
Error	18	2772.953	154.053		
Total	26	40115.160			

Week 3

Source	DF	SS	MS	F-Value	Probability
NAA (A)	2	8732.839	4366.419	17.0358	0.0001
Sucrose (B)	2	5250.050	2625.025	10.2416	0.0011
AB	4	5046.437	1261.609	4.9222	0.0074
Error	18	4613.560	256.309		
Total	26	23642.885			

Week 4

Source	DF	SS	MS	F-Value	Probability
NAA (A)	2	7735.414	3867.707	22.8444	0.0000
Sucrose (B)	2	3997.816	1998.908	11.8064	0.0005
AB	4	3430.779	857.695	5.0659	0.0065
Error	18	3047.520	169.307		
Total	26				

Appendix 11. ANOVA tables for effects of IBA and sucrose on rooting

Week 1

Source	DF	SS	MS	F-Value	Probability
IBA (A)	2	5277.962	2638.981	9.4272	0.0016
Sucrose (B)	2	3936.329	1968.164	7.0309	0.0055
AB	4	3679.182	99.796	3.2858	0.0345
Error	18	5038.771	279.932		
Total	26	17932.247			

Week 2

Source	DF	SS	MS	F-Value	Probability
IBA (A)	2	1341.383	670.691	1.8324	0.1886
Sucrose (B)	2	707.845	353.923	0.9670	
AB	4	1949.108	487.277	1.3313	0.2965
Error	18	6588.173	366.010		
Total	26	10586.510			

Week 3

Source	DF	SS	MS	F-Value	Probability
IBA (A)	2	637.262	318.631	1.0760	0.3619
Sucrose (B)	2	22.976	11.488	0.0388	
AB	4	1045.729	261.432	0.8828	
Error	18	5330.413	296.134		
Total	26	7036.380			

Week 4

Source	DF	SS	MS	F-Value	Probability
IBA (A)	2	563.116	281.558	2.3386	0.1251
Sucrose (B)	2	75.079	37.539	0.3118	
AB	4	81.984	20.496	0.1702	
Error	18	2167.153	120.397		
Total	26	2887.332			

Appendix 12. ANOVA table for effects of source of shoots on percent survival

Source	DF	SS	MS	F-value	Probability
Source of shoots	2	280.809	140.404	1.1396	0.3806
Error	6	739.240	123.207		
Total	8	1020.049			

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