Explants, Hormones and Sucrose Influence in vitro Shoot Regeneration and Rooting of Calla Lily (*Zantedeschia albomaculata* l. Spreng.) 'black magic'

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ABSTRACT

Zantedeschia is an important and rapidly expanding cut flower in Kenya today. A protocol for in vitro shoot regeneration of *Zantedeschia,* using tuber, leaf and shoot primordium explants on Murashige and Skoog (MS) (1962) basal salts, supplemented with 6-benzyalamino purine (BAP) or Kinetin is described. Of the four levels (0, 1, 2, and 4 mg/l) each of BAP and Kinetin, 2 mg/l BAP induced the highest number of shoots per explant (2.5) and the longest shoots (3.7 cm) on shoot explants after four weeks in culture. No response was observed on both leaf and tuber explants on all media tested. A 34-fold shoot multiplication rate was achieved in a culture period of 10 weeks. Rooting was tested using three levels (0, 1, and 2 mg/l) each of naphthalene acetic acid (NAA) and indole butyric acid (IBA) in combination with three levels (15, 20 and 30 g/l) of sucrose. The highest (84%) root induction in the shortest time (within one week) occurred in the medium containing 20 g/l sucrose + 1 mg/l IBA. However after four weeks, all shoots (100%) in this medium and the hormone-free media plus 15 to 30 g/l sucrose had rooted. Thus, this research established that shoot explants of *Zantedeschia albomaculata* cultivar Black Magic can be induced to produce high quality multiple shoots, using MS (1962) basal salts, supplemented with 2 mg/l BAP, 30 g/l sucrose and 0.8% agar. All the shoots can be readily and inexpensively rooted in growth regulator-free medium, containing 15 to 30 g/l sucrose, before transferring plantlets ex vitro.

1.0 INTRODUCTION

Calla lilies (*Zantedeschia* species) and their hybrids can be grown as commercial cut flowers in temperate and sub-tropical climates. Calla lily 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 most prevalent in southern

Africa, but it also extends north up to Nigeria (Funnell, 1993). The horticultural industry contributes significantly to the Kenyan economy, being the third leading foreign exchange earner, after tea and tourism. The cut flower industry constitutes the leading percentage of commercial horticulture in Kenya. In 2001, flowers earned Kshs. 12.7 billion, ahead of vegetable and fruit exports that earned Kshs. 8.4 and 2.5 billions, respectively (HCDA, 2001).

Zantedeschia is an important and rapidly expanding cut flower in Kenya. It is improving both in volume and value in the export market. Commercially, multiplication is achieved using seed and offsets, but only four selections are commercially produced from seed. Multiplication using offsets is slow. In addition, there has been a problem in obtaining disease-free calla lily planting materials, because continuously subdivided conventional tubers accumulate pathogens. This cut flower has not been extensively investigated through biotechnology, especially tissue culture in Kenya. Scanty information concerning regeneration of calla lily from different explants on different media appears in the literature. In Kenya, tissue culture techniques are not currently applied for clonal propagation of *Zantedeschia,* forcing growers to import clean starting materials for bulking. Importation increases the cost of producing *Zantedeschia.*

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). Many tissue culture responses are caused by interactions between the plant's genotype and the culture environment. The responses obtained in vitro can also be influenced by the way that stock plants are treated, as well as the environment in which they are grown. 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). The use of tissue culture is advantageous with valuable crops, because it can supplement conventional breeding by accelerating the clonal multiplication and release of new cultivars to growers. Moreover, efficient in vitro regeneration is a pre-requisite for genetic engineering of crops (Jain and Minocha, 2000). Using tissue culture as a rapid method to create an initial stock of *Zantedeschia* would provide the foundation for creating healthy stocks of selected clones in a commercially acceptable time.

This study, therefore, aimed at developing tissue culture techniques for calla lily in Kenya, using *Zantedeschia albomaculata* 'Black Magic' as an example. The specific objectives of the study involved determining effects of explants, hormones and sucrose on in vitro shoot regeneration and rooting. Three types of explants (leaf, tuber and shoot) were cultured on various levels of cytokinins, whereas resulting shoots were rooted in the presence of auxins in combination with three levels of sucrose.

2.0 MATERIALS AND METHODS

2.1 Explant Preparation

Tubers that were freshly harvested and air-dried in an open sheltered room for at least one week were obtained from the Sian-Agriflora (Kenya) Limited commercial flower farm. Some of the tubers were planted in containers in the greenhouse to supply leaf explants. Others were kept in the laboratory until buds produced small shoots that were then used as shoot explants. Initial surface-sterilization involved washing 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 and shoot tip explants were cut into cubes, measuring 5 mm x 5 mm x 5 mm, while leaf explants were cut into 5-mm diameter discs. Sterile explants were cultured in flasks, containing 25 ml of the initiation medium. This procedure was employed in all the experiments.

2.2 Tissue Culture Conditions and Experiments

The initiation medium consisted of MS (1962) basal salts, gelled with 0.8% agar. Sucrose and hormone concentration varied with the experiment. Media were prepared from stock solutions prepared for MS (1962) macronutrients, micronutrients, vitamins, iron-EDTA and hormones. The pH of all media was adjusted to 5.8 prior to autoclaving for 18 minutes at 121° C and 100 kPa. All cultures were incubated in a culture cabinet maintained at 27 ± 1° C and 16 hours of light.

In experiment I, 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 to determine the level that would best

stimulate shooting and subsequent shoot multiplication of calla lily cultivar Black Magic. Each treatment was assigned 6 explants of each kind, and replicated three times. The experiment was set up as a 4 x 3 factorial in a Completely Randomized Design for each type of hormone. Observations began 7 days after initiation of cultures and data was recorded for the number of shoots per explant and percentage of explants with shoots. Explants were subcultured every four weeks. The total number of shoots produced per cultured explant at the end of 3 culture passages was recorded for each treatment.

In experiment II, in vitro-regenerated shoots were cultured on full strength MS (1962) basal salts, augmented with IBA (0, 1 and 2 mg/l) or NAA (0, 1 and 2 mg/l) plus sucrose at three levels (15, 20 and 30 g/l) to determine the best rooting medium for calla lily cultivar Black Magic. Six shoots and three replications represented each treatment. The experimental layout for each hormone was a 3 x 3 factorial in a completely randomized design. Thus, the IBA and NAA effects were analyzed separately. Observations on root induction were conducted weekly. Data on time taken to rooting and percentage of rooted shoots after four weeks were recorded. Data were subjected to analysis of variance, and mean separation was performed using either the Least Significant Difference test or the Duncan's Multiple Range test.

3.0 RESULTS AND DISCUSSION

3.1 Effect of BAP and Kinetin on Shooting

The initial responses of explants included tissue browning and shoot elongation in shoot meristem explants (Table 1). Tissue browning was observed at the bases of explants within the first week, but it was not severe to kill or adversely affect the growth of explants. This condition, however, 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 in culture. This contamination was considered primary contamination, which occurs during the first two weeks in culture 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 at 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 performed (Taji and Williams, 1996). However, techniques to rid explants of contaminants must be developed to suit individual species and explants. Thus in our study, use of an additional disinfectant, containing 0.1% mercuric chloride reduced primary contamination to less than 10% in shoot explants and 2% in both tuber and leaf explants (data not shown).

During the first week, shoot explants elongated rapidly and produced one to two axillary shoots, although those in the medium without hormones stunted and regenerated no axillary shoots (Table 1).

Table 1: Effect of explant, BAP and Kinetin on shooting of calla lily (*Z.albomaculata)* 'Black Magic' after four weeks in culture

BAP (mg/l)	Type of explant	Percent shooting	Shoots per explant	Shoot length (cm)
0	Leaf	0	0	0
	Tuber	0	0	0
	Shoot	100	2	2
1	Leaf	0	0	0
	Tuber	0	0	0
	Shoot	100	2	3
2	Leaf	0	0	0
	Tuber	0	0	0
	Shoot	100	2	4
4	Leaf	0	0	0
	Tuber	0	0	0
	Shoot	100	2	3
Kinetin (mg/l)				
0	Leaf	0	0	0
	Tuber	0	0	0
	Shoot	100	1	1
1	Leaf	0	0	0
	Tuber	0	0	0
	Shoot	100	2	2
2	Leaf	0	0	0
	Tuber	0	0	0
	Shoot	100	2	2
4	Leaf	0	0	0
	Tuber	0	0	0
	Shoot	100	2	2

Shoot growth and elongation began after 3 to 4 days of initiation in the culture media. There was 100% shooting 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, however, darkened and deteriorated in culture.

Regeneration of leaf tissues is routinely used for propagation of 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,* harbouring an engineered plasmid (Billings et al. 1988). Park et al. (1995) reported regeneration of potato shoots from leaf tissues, but responses to published regeneration regimes showed cultivar specificity. Geier (1986), working with *Anthurium scherzerianum,* induced plantlets from spadix explants and later from leaves.

Efficient, reproducible and rapid in vitro regeneration systems are a pre-requisite 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. It is generally accepted that regeneration of plantlets from callus tissue frequently results in chromosome aberrations 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 levels tested. However, shoot regeneration has been achieved in other species, using tuber explants. Ng' (1988) obtained direct plant regeneration from tuber discs of *Dioscorea rotundata* (White yam), cultured on MS (1962) medium supplemented with 0.5 or 1 mg/l BAP. Complete formation of plantlets occurred within 3 months in culture. George (1985) also reported that potato tuber discs cultured on MS (1962) medium, supplemented with Nitsch and Nitsch (1955) organic addenda and other components, regenerated and developed normal plantlets after 8 weeks.

In the current research, once tuber and leaf explants failed to respond on both BAP and Kinetin media, the experimental design was changed to a one factor Completely Randomized Design and data were subjected to analysis of variance to compare the effect of BAP and Kinetin on shoot explants. The initial growth rates among treatments were similar until after two weeks when a significant difference in the number of shoots was

observed (Table 2). 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 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 media were obtained. After four weeks in the initial culture, the highest shoots per explant (2.3) were observed when the medium was supplemented with 4 mg/l BAP, compared to 1.8 in the best Kinetin (4 mg/l) concentration.

Table 2: Effect of BAP and Kinetin on shooting and shoot length of calla lily (*Z. albomaculata*) 'Black Magic'

	BAP (mg/l)[Z]				Kinetin (mg/l)				
Time	0	1	**2**	**4**	**0**	**1**	**2**	4	
(week)	Number of shoots per explant								LSD
1	1.0	1.5	1.6	1.7	1.0	1.1	1.3	1.4	NS
2	1.5^{bc}	1.8^{ab}	2.2^{a}	1.8^{ab}	1.2^{c}	1.3^{c}	1.3^{c}	1.5^{bc}	0.46
3	1.5^{c}	1.8^{abc}	2.5^{a}	2.4^{ab}	1.2^{c}	1.5^{c}	1.5^{c}	1.7^{bc}	0.77
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}	2.3^{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}	2.5^{bc}	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 rows are not significantly (NS) different, according to the DMRT at P = 0.05

Shoots obtained in 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 shoots are then of poor quality, with short stems, small internodes and difficult to separate. To overcome this problem, Rugini and Verma (1983) used two types of media for stage II of almond shoot tip cultures. The first incorporated 0.7 mg/l BAP and 0.1 mg/l NAA to induce shoot multiplication. The

second with a lower level of BAP (0.2 mg/l) and no auxins induced the shoots to elongate to a sufficient size for rooting.

Subculturing shoots every four weeks to fresh medium of the same composition essentially enhanced shoot proliferation in BAP media (data not shown). Few axillary shoots were obtained in a growth-regulator-free and Kinetin medium. The number of excisable shoots (0.5 to 1.5 cm) per explant was summed over 10 weeks and a concentration of 2 mg/l BAP promoted the highest multiplication fold (34 times), compared to the medium with 4 mg/l kinetin (3.5 times). The number of axillary shoots increased markedly in BAP media and this difference remained constant throughout the three subcultures.

These results are similar to those obtained by Clemente et al. (1991) in *Artemisia granatensis*, in which the highest shoot proliferation rate was achieved with 0.44 μM BA, although most of the axillary shoots were vitrified by the fourth subculture. Although elongation of the main shoot was greatest on the medium without BA, the number of axillary shoots increased markedly with BA treatments throughout the four subcultures. Debergh and Maene (1977) found that several axillary shoots and later adventitious shoots were obtained on *Pelargonium* if apices of individual buds developing on proliferating meristems were subcultured to fresh medium. In the current study, nearly all concentrations of BAP produced more axillary shoots than the Kinetin levels tested (Table 2). Axillary and adventitious shoot proliferation is stimulated after adding high cytokinin levels in the medium (Hartmann et al. 1997; George, 1993). From these results, probably higher than 4 mg/l Kinetin could be required for enhanced shoot proliferation in calla lily. In Geraldon wax, 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). It is apparent from the current experiment that optimum adventitious shoot formation on shoot explants of calla lily cultivar Black magic is promoted by subculturing.

3.2 Effect of NAA, IBA and Sucrose on Rooting

The rooting stage is the most critical step in the micropropagation of plant species if clonal mass production of material is to be achieved. 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 were found, when the effect of NAA and sucrose on rooting were analyzed weekly for four weeks (Table 3). In the first week, no roots formed in all NAA-augmented media (Table 3a). The highest rooting (80%) was achieved in the medium supplemented with 15 g/l sucrose with no NAA, compared to 69% and 67% in media containing 30 g/l and 20 g/l with no NAA, respectively.

Table 3: Effect of NAA IBA and sucrose on percentage in vitro rooting of calla lily *(Z. albomaculata)* Black Magic' shoots

Table 3(a)		Time in culture (weeks)[z]			
Sucrose (g/l)	**NAA (mg/l)**	**1**	**2**	**3**	**4**
15	0	80^{a}	93^{a}	100^{a}	100^{a}
20	0	67^{b}	87^{ab}	87^{ab}	100^{a}
30	0	69^{b}	93^{a}	100^{a}	100^{a}
15	1	0^{c}	7^{e}	40^{c}	65^{c}
20	1	0^{c}	64^{c}	76^{ab}	88^{ab}
30	1	0^{c}	70^{bc}	76^{ab}	76^{bc}
15	2	0^{c}	8^{de}	14^{d}	23^{d}
20	2	0^{c}	0^{e}	80^{ab}	88^{ab}
30	2	0^{c}	24^{d}	66^{b}	66^{c}
	LSD	37	21	27	13

Table 3(b)		Time in culture (weeks)			
Sucrose (g/l)	**IBA (mg/l)**	**1**	**2**	**3**	**4**
15	0	80^{ab}	93	100	100
20	0	80^{ab}	87	87	100
30	0	69^{ab}	93	100	100
15	1	27^{cd}	80	80	93
20	1	84^{a}	89	100	100
30	1	52^{bc}	67	92	92
15	2	17^{d}	56	83	94
20	2	52^{bc}	90	82	89
30	2	61^{ab}	79	86	86
	LSD	29	NS	NS	NS

[z]Values not followed by a letter or followed by the same letter within a column of each hormone are not significantly (NS) different, according to the LSD test at P = 0.05.

However in the second week, there was a significant interaction between NAA and sucrose, whereby 70% rooting occurred when 20 g/l sucrose was combined with 1 mg/l

NAA. This value was, however, lower than the 93% achieved with 0 mg/l NAA plus 15 g/l or 30 g/l sucrose (Table 3a).

By the end of three weeks, there was no significant difference among the media supplemented with 20 g/l sucrose + 1 mg/l NAA, 15 g/l sucrose + 2 mg/l NAA and 30 g/l sucrose + 1 mg/l NAA. These were significantly different from 0 mg/l NAA plus 15 g/l or 30 g/l sucrose, which had achieved 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 formed roots in the medium augmented with 20 g/l sucrose + 1 mg/l NAA (Table 3a). Thus, rooting in hormone-free media was better than in NAA-augmented media. Microcuttings of *Brassica oleracea* Capitata group also rooted readily on growth-regulator-free medium (Lillo and Shahin, 1986).

Although most species root satisfactorily with the addition of 20 to 30 g/l sucrose to the rooting medium, there was no significant difference in percent rooting in media supplemented with 15, 20 or 30 g/l sucrose alone, suggesting that sucrose did not directly influence rooting of *Zantedeschia* shoots. This proved that any of the three levels of sucrose 15, 20 and 30 g/l tested is suitable for rooting shoots of calla lily cultivar Black Magic. Page and Visser (1989) have reported that a decrease of either the MS (1962) components or the 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.

Most shoots cultured in the medium supplemented with or without IBA rooted in 4 to 8 days (Table 3b). 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 or 20 g/l sucrose plus no IBA, respectively.

From the second week in culture, no significant difference in rooting percentage was observed among the rooting media augmented with 15, 20 or 30 g/l sucrose alone and those with IBA (Table 3b). Nevertheless, the medium with 15 and 30 g/l sucrose alone induced the highest (93%) rooting percentage in the second week, compared to 90% and 89% in the media augmented with 20 g/l sucrose + 2 mg/l IBA and 20 g/l sucrose + 1 mg/l IBA, respectively (Table 3b).

By the end of three weeks, the highest rooting percentage (100%) was achieved in the media augmented with 15 or 30 g/l plus no IBA, respectively, and that with 20 g/l sucrose + 1 mg/l IBA. The lowest rooting (80%) was achieved in the medium augmented with 15 g/l sucrose + 1 mg/l IBA (Table 3b). After 4 weeks in rooting media, most shoots in all IBA-augmented media and the control had developed roots, 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 resulted in media with 15 g/l sucrose + 2 mg/l IBA and 15 g/l + 1 mg/l IBA, respectively. From these results, it can be deduced that there was an interaction between sucrose and IBA concentrations on the rooting of *Zantedeschia albomaculata* 'Black Magic' shoots.

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. Although roots formed in the absence of auxins, addition of IBA increased rooting better than NAA (Table 3). These results are similar to those obtained in Chinese cabbage, whereby roots formed in the absence of auxins, but addition of IBA or NAA increased the percentage of rooting (Kee et al. 1987).

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 in IBA-augmented media were long, thin and branched with root hairs, compared to short, thick and without root hairs in NAA-augmented media. These results are similar to those obtained 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 Kee et al. (1987).

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 + 2 mg/l IBA versus 30 g/l sucrose + 2 mg/l NAA, respectively. This and previous findings proved that IBA is a better rooting hormone than NAA for 'Black Magic' shoots. Contrary to these results, Clemente et al. (1991) reported that incorporation of IBA at 0.49 to 14 μM or NAA at 0.53 to 16 μM into the basal medium for 3 days did not enhance either the rooting percentage (70%) or the number of roots per culture (3.5) relative to those obtained in controls. The presence of IAA during the entire culture period decreased the rooting

percentage to 50% at 0.57 μM and 0% at 17 μM, whereas roots did not form with IBA and NAA at any of the levels tested. However, the overall best rooting medium in the current study contained 15 to 30 g/l sucrose and no hormones.

In conclusion, it is clear from the current study that *Zantedeschia albomaculata* 'Black Magic' can be induced in vitro to produce high quality multiple shoots from shoot-bud explants, using full-strength MS (1962) basal salts, supplemented with 30 g/l sucrose and 2 mg/l BAP. Root induction is best in a hormone-free medium, supplemented with 15 g/l to 30 g/l sucrose.

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