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Eucomis zambesiaca baker: Factors affecting *in vitro* bulblet induction

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Abstract

Eucomis species having considerable horticultural potential are used in African traditional medicine to treat various ailments. The effects of environmental and physiological parameters on the initiation and growth of bulblets using leaf explants were investigated. These included the effect of temperature (10, 15, 20, 25 and 30 °C), photoperiod (8 h light, 16 h light, continuous light and continuous dark), carbohydrates (sucrose, fructose and glucose) at different concentrations and combinations as well as various plant growth regulators; gibberellic acid (GA_3), indole-3-butyric acid (IBA), napthaleneacetic acid (NAA), N^6-benzyladenine (BA), zeatin and others. Liquid shake and liquid static cultures versus solid cultures were investigated. Maximum number of bulblets per leaf explant was obtained at 20 °C, with an average of 3 bulbs per leaf explants and a bulblet mass of 57 mg. An 8 h light cycle produced 1.38 bulbs per leaf explant, at a mass of 42 mg. Fructose at 3% produced an average of 1.18 bulbs per leaf explant, 3.39 mm wide and weighing 56.6 mg. Of the plant growth regulators, 4.90 μM IBA was found to be the optimum treatment for bulblet induction, with an average bulb diameter of 4.36 mm and a mean bulblet mass of 79.07 mg. Liquid shake cultures exhibited poor growth while bulblet, leaf and root growth was improved in liquid static cultures. Successful micropropagation from leaf explants established that leaf explants can be used as an alternative explant source to bulbs. This protocol allows for the fast and economic mass propagation of *Eucomis* plants.

Keywords: Auxin; Carbohydrates; Cytokinin; Hyacinthaceae; Photoperiod

1. Introduction

Trade in medicinal plants is an important part of the regional economy in South Africa with over 700 plant species being traded. It is estimated that there are 27 million indigenous people who use medicinal plants in South Africa (Mulholland and Drewes, 2004). Consequently demand often exceeds supply with regards to certain species. The indigenous bulbous plants that are of importance to traditional healers mainly belong to the Amaryllidaceae and Hyacinthaceae (Louw et al., 2002). The bulbs of *Eucomis* (Hyacinthaceae) species are of great value in Zulu, Xhosa and Tswana traditional medicine. This is largely due to their anti-inflammatory properties. As a result, the bulbs are harvested in great numbers from the wild for trade in South Africa's traditional medicinal markets.

Eucomis species are also highly ornamental and are used for gardening, cut-flowers and as flowering pot-plants. The bulb produces densely packed flower spikes which vary in colour from white to a yellowish-green and these flowers have a vase life of several weeks. Temperature and light play pivotal roles in the tissue culture environment influencing many responses of the cultured plants. Temperature is a natural regulator of plant growth and morphogenesis. It not only regulates growth rates but also the transition between various vegetative and reproductive phases during development (Ascough et al., 2008a). Light is one of the most important environmental factors in tissue culture and the effect it has on explants, can vary greatly. It has been reported that there are three categories of plant response to photoperiod with respect to storage organ formation. These are: induction of storage organs inhibited by darkness, induction of storage organs promoted by darkness and lastly, induction of storage organs by both light and darkness (Ascough et al., 2008b; Jacobs et al., 1992; Kim et al., 1981;

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Niimi and Onozawa, 1979; Slabbert and Niederwieser, 1999; Steinitz and Yahel, 1982).

Sucrose is considered to be the best source of carbon for *in vitro* tissue culture (George and Sherrington, 1984). It is usually hydrolysed partially or completely in the medium into the monosaccharides, glucose and fructose, which are taken up by the plant tissues. Relatively few studies have been conducted on the effect of different carbohydrate types and concentrations on bulb formation and growth of bulbous plants. Studies in which the sucrose concentration was varied are few and include those on *Lilium* and *Lachenalia* species (Niimi and Onozawa, 1979; Takayama and Misawa, 1979; Van Rensburg and Vcelar, 1989).

Plant growth regulators are the critical media components in determining the developmental pathway of the plant cells, however, there is considerable difficulty in predicting the effects of these plant growth regulators in tissue culture. Environmental factors such as temperature, light and day length interact with plant growth regulators to cause developmental responses in plants (Davies, 1987).

A number of plant tissue culture techniques have been developed for propagating ornamental species and some of these techniques are being used for large scale commercial propagation. One of these techniques is liquid culture. Liquid culture is a simple, easy, rapid and economical method for the propagation of bulbous plants (Ascough and Fennell, 2004; Bergoñón et al., 1992; Mehrotra et al., 2007). Large quantities of plants of medicinal, ornamental and ecological interest can be obtained by using this method.

In vitro propagation of *Eucomis* has focused on *Eucomis autumnalis* (Mill.) (Taylor and Van Staden, 2001a,b,c). *Eucomis zambesiaca* Baker. has not been micro-propagated before. Therefore the aim of this study was to develop a protocol to optimize the production of bulblets of *Eucomis zambesiaca in vitro* and to use this protocol in the production of other species in the Hyacinthaceae. Several factors affect the formation of bulblets *in vitro*. These include: the genetic makeup of the plant, culture conditions and supplements added to the culture medium (Ascough et al., 2008a). This study investigated the effect of temperature (10, 15, 20, 25 and 30 °C), photoperiod (8 h light, 16 h light, continuous light and continuous dark), sucrose, fructose and glucose ratios as well as the effect of various auxins and cytokinins on *in vitro* bulblet induction in *Eucomis zambesiaca*.

2. Materials and methods

Cultures of *Eucomis zambesiaca* Baker. plants were established using the protocol of Taylor and Van Staden (2001a). The cultures were multiplied and subcultured on Murashige and Skoog (1962) medium (MS medium) supplemented with 100 mg/L *myo*-inositol, 30 g/L sucrose and solidified with 8 g/L agar. The established plants were used to determine the effect of various factors on bulb induction.

Leaf material was placed such that either their abaxial or adaxial surfaces were in contact with the solid MS medium. The supplemented MS medium was used without the addition of plant growth regulators. The pH of the medium was adjusted to 5.8 with diluted KOH before autoclaving at 121 °C and 103 kPa for 20 min. Five pieces of uniform leaf material (1 cm length) were placed on 40 mL medium in each culture bottle (6 cm diameter, 10 cm high), eight bottles per treatment. Bottles were incubated in growth chambers (Conviron) which contained Osram L58W/640 cool white fluorescent bulbs. The Conviron had a light intensity range between 70 and 90 μmol/m^2/s. Cultures were incubated in growth chambers at temperatures of 10, 15, 20, 25 and 30 °C with a 16 h light photoperiod.

The cultures were maintained in growth chambers at 20 °C with varying photoperiods. The photoperiods tested were continuous light, continuous dark, 16 h light and 8 h light.

Leaves from the *in vitro* plants were cut into sections (1 cm length) and placed in culture bottles on 40 mL standard MS medium with 100 mg/L *myo*-inositol and solidified with 8 g/L agar. Different carbohydrate types (sucrose, glucose and fructose at concentrations 0, 10, 30, 60, 90 and 120 g/L) as well as combinations with each other (glucose/fructose 10/20, 15/15, 20/10, 30/30 and 45/45 g/L) were tested. The culture bottles were placed in growth chambers with a 16 h light photoperiod and a temperature of 20 °C.

Leaf explants (1 cm length) from previously established *in vitro* plantlets were inoculated onto 40 mL MS basal medium containing various plant growth regulators; napthaleneacetic acid (NAA), indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), 2,4-dichlorophenoxy acetic acid (2,4-D), phenylacetic acid (PAA), N^6-benzyladenine (BA), N^6-isopentenyladenine (iP), zeatin, *meta*-topolin (*m*T), gibberellic acid (GA_3), GA_4 and GA_7 gibberellin mixture (GA_{4+7}), abscisic acid (ABA), methyljasmonate (MeJA) and paclobutrazol (PAC) at various concentrations (see Table 4). The culture bottles were placed in growth chambers with a 16 h light cycle and a temperature of 20 °C.

For liquid culture, pieces of uniform leaf material (1 cm length) or 5 individual shoots, from previously established cultures, were placed in 100 mL Erlenmeyer flasks containing 40 mL liquid MS medium. The medium was supplemented with 100 mg/L *myo*-inositol and 30 g/L sucrose. No agar or plant growth regulators were added. The pH of the medium was adjusted to 5.8 with diluted KOH before autoclaving at 121 °C and 103 kPa for 20 min. Eight Erlenmeyer flasks containing leaf explants and eight containing shoots were placed on a rotary shaker at 100 rpm, while 16 flasks (8 containing leaves and 8 containing shoots) were placed on a shelf in a growth room. Leaf explants (5 pieces per jar) were also placed in culture bottles (6 cm diameter, 10 cm high) on 40 mL solid MS. The cultures were grown at 25±2 °C under a 16 h light cycle. The growth room had Osram L58W/640 cool white florescent light bulbs with an average light intensity of 74.4 μmol/m^2/s.

After 3 months, bulblets were removed and bulb mass and diameter were recorded. Data collected were subjected to one-way analysis of variance (ANOVA). Means were separated using Duncan Multiple Range Test (DMRT) at $p \leq 0.05$. Data analysis was carried out using SPSS version 15.0.

After *E. zambesiaca* bulblets were successfully grown *in vitro,* they were transferred to trays containing vermiculite and

placed in a mist-house. After 2 months the plantlets were transplanted to pots containing a sand:soil mixture of 1:1 and moved into a greenhouse.

3. Results and discussion

Growth of the leaf explants was observed after two to three weeks in culture with the appearance of small white globular shaped protuberances. After a further two months, the white protuberances turned green and developed into bulblets.

Table 1 shows the results for bulblet formation at different temperatures. A temperature of 20 °C produced the highest bulblet induction (Table 1). The lowest induction was obtained from the extreme temperatures of 10, 15 and 30 °C. At 20 °C bulblet number and bulblet mass were significantly higher. This indicates that *Eucomis zambesiaca* does not grow well *in vitro* under either a low or a high temperature, but rather at a moderate temperature.

At 20 °C the bulblets that formed had smaller leaves compared to the control grown at 25 °C, although, the number of bulblets per explant was higher than at any other temperature tested (Table 1). These results are in agreement with Takayama and Misawa (1979) and Yamagishi (1998) who both studied the effect of temperature on *Lilium* species and found 20 °C to be the optimum temperature for bulblet formation.

Fewer bulblets formed at 25 °C than 20 °C. It could be beneficial to initiate bulblet induction at 20 °C and then transfer them to 25 °C for growth, so that plantlet size can increase. Kulkarni et al. (2005) found that with *Albuca pachychlamys* (Hyacinthaceae) *ex vitro* seedling survival was higher at 10 and 15 °C, however, seedling and bulblet mass was greatest at 25 °C. So it was suggested that *A. pachychlamys* should be grown at low temperatures for a few days and then transferred to higher temperatures. This could possibly be beneficial for *E. zambesiaca* which also belongs to the Hyacinthaceae family.

Abaxially- and adaxially-orientated explants produced similar numbers of bulblets at all temperatures except 20 °C, where abaxially-orientated explants produced more (Table 1). Similarly, Leshem et al. (1982) and Taylor and Van Staden (2001a) found that abaxial orientation produced more bulblets or shoots. Explant orientation was tested in all experiments carried out for temperature and other effects (light, carbohydrates and hormones). As no significant differences were observed, the results for the other factors tested represent combined data for abaxially- and adaxially-orientated explants.

In some cases, a high bulblet induction treatment may produce small bulblets. Conversely a few, large bulblets may be produced in other treatments. There were significantly higher bulblets per explant and higher bulblet mass at 20 °C. Therefore, in all subsequent experiments, culture bottles were placed in growth chambers at 20 °C.

Continuous darkness completely inhibited bulblet induction (Table 2). The leaf segments were achlorophyllous and no response or differentiation was observed. The remaining photoperiods all induced bulblet formation. An 8 h light cycle produced the most bulblets per explant (1.38) as well as the heaviest bulbs (42.05 mg) (Table 2). Continuous light resulted in the largest bulbs (3.41 mm). However, these differences were not significant.

Other studies on bulblet induction have shown an opposite trend. For example, Economou and Read (1987) found that continuous darkness promoted bulblet regeneration and production in *Hyacinthus orientalis* bulb scale segments as well as increased bulb number and size in scale segments of *Lilium longiflorum*. They showed that a 16 h light photoperiod suppressed bulb formation in *L. longiflorum*. From the *E. zambesiaca* results it was shown that continuous darkness completely inhibited bulblet initiation. Light was needed to induce bulblets, however, the duration of light (8, 16 and 24 h) did not have a significant effect.

De Capite (1955), Leshem et al. (1982) and Kromer (1989) all obtained growth in both light and dark conditions, however, explants grown in the dark did not grow as well and produced fewer bulbs. Even though growth was reduced in continuous darkness, the explants did respond. This is again in contrast to our results. It is possible that the results obtained for *E. zambesiaca* differ from all the previous work carried out, due to the type of explant used or it could be due to the specific plant and species used. Further studies on light quality and light intensity on *E. zambesiaca* need to be investigated to optimize the true potential of light.

Table 3 shows the results for bulblet induction of different carbohydrate types and concentrations. The number of bulblets produced per leaf explant decreased as the concentration of the carbohydrate increased after 30 g/L. Little shoot and bulblet growth was observed at 60 g/L sucrose or fructose (Table 3). Higher concentrations of sucrose, fructose and glucose reduced

Table 1
Effect of temperature and explant orientation on bulblet induction per leaf explant and bulblet mass of *E. zambesiaca*.

Temperature (°C)	No. of bulblets per explants		Bulblet mass (mg)	
	Abaxial	Adaxial	Abaxial	Adaxial
10	0.90±0.26^{b}	0.50±0.24^{c}	13.08±4.54^{b}	10.91±4.12^{c}
15	0.65±0.32^{b}	0.25±0.15^{c}	30.29±12.93^{b}	10.58±8.14^{c}
20	3.00±0.62^{a*}	2.20±0.27^{a}	54.77±6.94^{a}	60.19±7.80^{a}
25	1.65±0.29^{b}	1.45±0.26^{b}	26.19±3.90^{b}	43.87±7.47^{b}
30	0.95±0.43^{b}	0.50±0.13^{c}	13.68±5.52^{b}	14.69±3.74^{c}

Mean values of the same orientation (abaxial/adaxial) with the same letter are not significantly different ($p \leq 0.05$). Asterisk indicates significant difference between abaxial/adaxial orientation ($p \leq 0.05$).

Table 2
Effect of four photoperiods on bulblet induction in leaf explants from *E. zambesiaca*.

Photoperiod	No. of bulblets per explant	Bulblet diameter (mm)	Bulblet mass (mg)
8 h light	1.38±0.18^{a}	3.38±0.17^{a}	42.05±5.48^{a}
16 h light	1.08±0.21^{a}	3.25±0.29^{a}	38.75±7.99^{a}
Continuous light	1.13±0.20^{a}	3.41±0.27^{a}	35.31±5.97^{a}
Continuous dark	0.00±0.00^{b}	0.00±0.00^{b}	0.00±0.00^{b}

Mean values in the same column followed by different letters are significantly different ($p \leq 0.05$).

Table 3
Effect of carbohydrate type and concentration on bulblet induction of *E. zambesiaca* leaf explants.

Carbohydrate	(g/L)	No. of bulblets per explant	Bulblet diameter (mm)	Bulblet mass (mg)
Control	0	0.16±0.07^{cde}	1.69±0.68^{def}	15.50±6.36^{efghi}
Sucrose	10	0.58±0.15^{b}	2.08±0.32^{cdef}	16.31±3.44^{defgh}
	30	0.60±0.22^{b}	2.42±0.41^{abcd}	23.40±6.92^{def}
	60	0.03±0.03^{de}	0.29±0.29^{gh}	2.00±2.00^{hi}
	90	0.05±0.03^{de}	0.73±0.49^{gh}	4.50±3.04^{hi}
	120	0.05±0.05^{de}	0.31±0.31^{gh}	2.81±2.81^{hi}
Fructose	10	0.50±0.13^{b}	2.28±0.38^{bcde}	25.64±6.88^{de}
	30	1.18±0.27^{a}	3.39±0.30^{a}	56.61±9.10^{a}
	60	0.25±0.11^{bcde}	1.11±0.44 ^{fg}	9.58±4.04^{fghi}
	90	0.03±0.03^{de}	0.34±0.34^{gh}	1.38±1.38^{hi}
	120	0.00±0.00^{e}	0.00±0.00 ^{h}	0.00±0.00^{i}
Glucose	10	0.45±0.07^{bc}	3.02±0.25^{abc}	29.20±7.04 ^{cd}
	30	0.50±0.08^{b}	3.26±0.34^{ab}	40.99±7.66^{bc}
	60	0.35±0.05^{bcde}	2.66±0.15^{abcd}	30.71±3.36 ^{cd}
	90	0.28±0.08^{bcde}	2.23±0.34^{bcde}	20.38±4.20^{defg}
	120	0.10±0.08^{de}	0.68±0.46^{gh}	5.96±4.63^{ghi}
Glucose/Fructose	10/20	0.50±0.05^{b}	2.71±0.14^{abcd}	31.85±3.32 ^{cd}
	15/15	0.38±0.07^{bcd}	1.83±0.27^{def}	11.88±1.91^{efghi}
	20/10	0.45±0.07^{bc}	3.36±0.24^{a}	46.27±6.86^{ab}
	30/30	0.33±0.06^{bcde}	1.86±0.29^{def}	13.50±2.55^{efghi}
	45/45	0.25±0.08^{bcde}	1.34±0.42^{efg}	10.83±3.94^{efghi}

Mean values in the same column followed by the same letter(s) are not significantly different ($p \leq 0.05$).

bulblet growth. At 120 g/L fructose, the leaf explants turned brown and no response was seen. Fructose was the best carbohydrate for bulblet induction in *E. zambesiaca*, in particular 30 g/L fructose. A possible reason for this could be that *E. zambesiaca* may have a different sugar sensing and signaling system. This means that the plant senses or recognizes fructose better than the other carbohydrates. Another reason could be that when the plant tissue is presented with different carbohydrates such as sucrose, fructose and glucose, it selects/prefers fructose as it does not have to break down sucrose. By selecting fructose the plant eliminates the energy needed to hydrolyze sucrose and provide energy for growth (Smeekens, 2000).

Bach et al. (1992) studied the effects of sucrose, fructose and glucose on bulblet formation on *Hyacinthus orientalis* leaf explants. Media supplemented with fructose produced more bulblets than the other carbohydrates, and more bulblets were produced on media with a concentration of 30 g/L carbohydrate as opposed to a 60 g/L concentration. The results from the experiments carried out on *E. zambesiaca* looking at various carbohydrates, concentrations and combinations of carbohydrates are in agreement with the data obtained by Bach et al. (1992).

After 30 g/L fructose, 30 g/L glucose and a 1:2 and 2:1 glucose:fructose ratio gave the next best results. There was no significant difference between 30 g/L fructose and 2:1 glucose: fructose, for both mean bulb diameter (3.39 and 3.36 mm) and mean bulb mass (56.6 and 46.3 mg). These results are in contrast to those of Langens-Gerrits et al. (2003) who tested various sugars (glucose, fructose, mannose and sucrose) on lily. None of the sugars, nor a combination of glucose and fructose exceeded sucrose in promoting the growth of bulblets. The mass per bulblet increased with increasing sucrose concentration. This was not the case with bulblets of *E. zambesiaca* – when the carbohydrate concentration increased – mean bulblet mass decreased.

Although fructose was optimum for *E. zambesiaca*, another species (*E. autumnalis*) grew best in sucrose (Taylor and Van Staden, 2001b). Thus, the capacity of plant tissues to utilize carbohydrates varies between species and even explants (Mezzetti et al., 1991). It also depends on the ability of the tissue to absorb, transport and metabolize the carbohydrates.

For bulblet induction, 2,4-D at a concentration of 4.52 μM produced significantly more bulbs than any other treatment (Table 4). The explants swelled and became yellowish-brown. Although the number of bulbs was highest on 2,4-D-containing medium, they were small in diameter (1 to 3 mm) compared to 4.90 μM IBA. However, when 2,4-D was compared with the control (2.28 mm) the sizes of the bulbs were not much bigger (Table 4). The widest and largest bulblets were produced by 4.90 μM IBA. Treatments of 5.37 μM NAA, 24.60 μM IBA and 11.42 μM IAA produced better results than the control (Table 4).

The best results were produced by 5.37 μM NAA and 4.90 μM IBA. These bulbs were bright green in colour, had strong, healthy leaves and the roots were long and well established. Fewer bulbs were produced per explant on these treatments although the bulblets formed were larger than those formed on 2,4-D. 2,4-D produced the most bulbs (Table 4). Better bulblet induction results were obtained with IBA-containing medium than with other plant growth regulators. This could result from the fact that IBA tends to be broken down in the media and rapidly metabolized within plant tissues (Gaspar et al., 1996).

Dabrowski et al. (1992) found similar results for *Lilium* bulb-scales with regards to auxins producing good results for bulblet formation, however, the addition of both auxins and cytokinins to the medium increased the regeneration ability of explants more. Yi et al. (2002) found that medium with IBA had the highest regeneration and growth rate of bulblets in *Hyacinthus orientalis* cv. Carnegie compared to IAA. These results are in agreement with our data, where we also found that there was an increase in bulblet number with IBA.

Adventitious bulb formation from the bulb scale explants of hyacinth was stimulated when IAA was added to the culture medium. An increase in IAA increased the number of bulbs produced as well as bulb mass (Pierik and Steegmans, 1975; Rudnicki, 1979). For *E. zambesiaca*, both 11.42 and 28.54 μM IAA produced better results than 5.71 μM. Not only were more bulbs produced per leaf explant, but the size of the bulblets were larger at the higher concentrations.

Of the cytokinins, BA and zeatin produced more bulbs compared with the number of bulbs produced from iP and *m*T treatments (Table 4). Of the BA treatments, a concentration of 22.19 μM produced more bulblets than 4.44 and 8.87 μM. At 22.19 μM BA, the average number of bulblets per explant was 0.73 and the bulblets had a mean diameter of 2.1 mm and mean mass of 15.85 mg. Takayama et al. (1991) found that high

Table 4
Effect of various plant growth regulators and concentrations on the average number, size and weight of bulblets produced on *E. zambesiaca* leaf explants.

PGR	Conc. (μM)	No. of bulblets per explants	Bulblet diameter (mm)	Bulblet mass (mg)
Control	0	0.35 ± 0.063^{ghijklm}	2.28 ± 0.094^{cdefghij}	16.94 ± 1.85^{defghi}
NAA	5.37	1.13 ± 0.164^{bc}	3.51 ± 0.316^{ab}	51.99 ± 11.00^{b}
	10.74	0.78 ± 0.175^{cdef}	2.68 ± 0.193^{bcdefg}	29.12 ± 5.26^{cde}
	26.85	0.43 ± 0.080^{fghijklm}	2.41 ± 0.164^{bcdefghi}	18.68 ± 4.98^{cdefghi}
2,4-D	4.52	1.53 ± 0.501^{a}	2.76 ± 0.124^{bcdef}	27.77 ± 2.79^{cdef}
	9.05	0.63 ± 0.139^{efghi}	2.36 ± 0.117^{cdefghij}	21.15 ± 2.26^{cdefgh}
	22.62	0.25 ± 0.091^{hijklm}	1.54 ± 0.467^{ghij}	9.63 ± 3.38^{efghi}
IAA	5.71	0.38 ± 0.116^{fghijklm}	1.83 ± 0.575^{defghij}	22.34 ± 9.36^{cdefg}
	11.42	0.65 ± 0.091^{efgh}	2.95 ± 0.258^{bcd}	37.99 ± 8.07^{bc}
	28.54	0.60 ± 0.125^{efghij}	2.52 ± 0.484^{bcdefgh}	30.28 ± 8.41^{cde}
IBA	4.90	1.08 ± 0.106^{bcd}	4.36 ± 0.390^{a}	79.07 ± 17.35^{a}
	9.80	0.93 ± 0.239^{bcde}	2.84 ± 0.455^{bcde}	34.14 ± 8.23^{bcd}
	24.60	1.28 ± 0.160^{ab}	3.51 ± 0.190^{ab}	50.14 ± 8.03^{b}
PAA	7.34	0.25 ± 0.082^{hijklm}	1.28 ± 0.416^{ijk}	12.04 ± 4.92^{efghi}
	14.68	0.28 ± 0.065^{hijklm}	2.08 ± 0.308^{cdefghij}	20.35 ± 3.61^{cdefghi}
	36.71	0.20 ± 0.085^{ijklm}	1.15 ± 0.446^{jk}	10.25 ± 4.31^{efghi}
BA	4.44	0.45 ± 0.098^{fghijkl}	1.84 ± 0.158^{defghij}	12.94 ± 1.81^{efghi}
	8.87	0.38 ± 0.110^{fghijklm}	1.76 ± 0.409^{efghij}	14.53 ± 3.88^{defghi}
	22.19	0.73 ± 0.131^{defg}	2.10 ± 0.339^{cdefghij}	15.85 ± 3.86^{defghi}
Zeatin	4.56	0.55 ± 0.050^{efghijk}	1.82 ± 0.106^{defghij}	14.53 ± 1.60^{defghi}
	9.12	0.63 ± 0.059^{efghi}	1.59 ± 0.062^{fghij}	10.02 ± 0.90^{efghi}
	22.81	0.40 ± 0.085^{fghijklm}	1.52 ± 0.235^{ghij}	11.21 ± 1.80^{efghi}
iP	4.92	0.20 ± 0.065^{ijklm}	1.18 ± 0.353^{jk}	7.94 ± 2.47^{fghi}
	9.84	0.33 ± 0.075^{ghijklm}	1.95 ± 0.072^{cdefghij}	14.47 ± 0.76^{defghi}
	24.60	0.53 ± 0.075^{efghijk}	1.92 ± 0.068^{cdefghij}	13.02 ± 1.54^{efghi}
*m*T	4.14	0.35 ± 0.033^{ghijklm}	1.66 ± 0.075^{efghij}	10.00 ± 1.33^{efghi}
	8.29	0.55 ± 0.118^{efghijk}	1.46 ± 0.222^{hijk}	8.39 ± 1.59^{fghi}
	20.73	0.53 ± 0.106^{efghijk}	1.60 ± 0.243^{fghij}	11.41 ± 2.17^{efghi}
GA_3	2.89	0.40 ± 0.120^{fghijklm}	2.81 ± 0.684^{bcde}	38.35 ± 14.17^{bc}
	5.77	0.28 ± 0.037^{hijklm}	3.03 ± 0.334^{bc}	23.81 ± 7.62^{cdefg}
	14.43	0.18 ± 0.059^{jklm}	1.74 ± 0.566^{efghij}	10.38 ± 5.62^{efghi}
GA_{4+7}	2.11:0.908	0.23 ± 0.070^{hijklm}	1.51 ± 0.361^{ghij}	6.73 ± 1.86^{ghi}
	4.22:1.81	0.05 ± 0.033^{lm}	0.39 ± 0.258^{kl}	1.38 ± 0.94^{hi}
	10.55:4.54	0.00 ± 0.000^{m}	0.00 ± 0.000^{l}	0.00 ± 0.00^{i}
ABA	3.78	0.33 ± 0.075^{ghijklm}	1.40 ± 0.218^{hijk}	8.35 ± 1.78^{fghi}
	7.57	0.20 ± 0.053^{ijklm}	1.22 ± 0.291^{ijk}	7.13 ± 1.92^{fghi}
	18.92	0.30 ± 0.065^{ghijklm}	1.22 ± 0.274^{ijk}	5.88 ± 1.45^{ghi}
MeJa	4.45	0.23 ± 0.070^{hijklm}	1.81 ± 0.574^{defghij}	17.56 ± 8.37^{defghi}
	8.91	0.20 ± 0.065^{ijklm}	1.65 ± 0.492^{efghij}	10.56 ± 3.53^{efghi}
	22.25	0.15 ± 0.033^{klm}	1.62 ± 0.368^{fghij}	10.38 ± 2.78^{efghi}
PAC	3.40	0.50 ± 0.038^{fghijk}	2.26 ± 0.134^{cdefghij}	19.79 ± 2.59^{cdefghi}
	6.80	0.28 ± 0.053^{hijklm}	2.02 ± 0.333^{cdefghij}	21.13 ± 5.06^{cdefgh}
	17.00	0.18 ± 0.059^{jklm}	1.61 ± 0.555^{fghij}	18.75 ± 8.26^{cdefghi}

Mean values in the same column followed by the same letter(s) are not significantly different ($p \leq 0.05$).
Ratio of GA_4:GA_7 is 70:30.

concentrations of BA stimulated the formation of bulbs in *H. orientalis*. Although more bulblets were produced on BA-containing than *m*T-containing media, shoot production in other species was enhanced by *m*T (Bairu et al., 2007). After 22.19 μM BA, the following, 8.89 μM BA, 4.56 μM zeatin and 9.84 μM iP, had the heaviest bulblet masses of 14.53, 14.53 and 14.47 mg respectively.

The gibberellins (GA_{4+7}) produced fewest bulblets, while 2.89 μM GA_3 produced the most bulblets of the GA treatments (Table 4). Although few bulblets formed on medium with GA_3, the bulblets that did form were significantly larger than the control (1 to 3 mm) and had long thin leaves. At a concentration of 10.55:4.54 μM GA_{4+7}, bulblet formation was inhibited. Pierik and Steegmans (1975) reported that bulblet growth in hyacinth was reduced with an increase in GA_3 concentration. This was also true for *E. zambesiaca* as increasing concentration decreased bulblet regeneration and growth.

Hyacinth regeneration and bulblet growth were decreased proportionally to an increase in the concentration of ABA (Pierik and Steegmans, 1975). Increasing ABA concentrations was also found to decrease bulblet size in *E. zambesiaca* (Table 4). MeJA increased bulblet induction and size in *Allium sativum* cv. Ptuj (Ravnikar et al., 1993) and *Narcissus triandrus* (Santos and Salema, 2000), it did not however, stimulate bulblet formation in *E. zambesiaca* at any concentration (Table 4).

Growth retardants, such as PAC, are often used in tissue culture as they are useful in preventing leaf growth and shoot vitrification (Ilczuk et al., 2005). Within the PAC treatments,

3.40 μM PAC was better than 6.80 and 17.00 μM in terms of mean number of bulbs per explant and mean bulb diameter. The heaviest bulbs were formed on medium supplemented with 6.80 μM PAC. However, when PAC treatments are compared to the control, only 3.40 μM PAC produced more bulbs per explant but all PAC treatments produced heavier bulbs than those grown on the control medium. All the bulblets that developed on medium containing PAC had fewer leaves which were short and stunted.

The same composition of growth regulators in the culture medium may cause different reactions in different species and cultivars, or similar reactions may be seen when sections of different organs are placed on the same medium. These variations are due to the fact that different species and sections of various organs, differ in their ability to produce endogenous growth substances or they differ in their content at the time of isolation (Hempel, 1979). Variations could also be a result of the species or section of organ's ability to recognize and take up exogenous plant growth regulators as well as their ability to transport, metabolize and respond to the exogenous plant growth regulators. Therefore, it is not always possible to dictate a particular concentration or plant growth regulator to be used.

Plants formed in liquid culture were larger than those grown on solid medium (Table 5). Thus, it may be possible to harvest healthy *E. zambesiaca* bulblets using liquid culture compared to explants grown on solid MS medium. Leaf and shoot explants grown in liquid static culture produced 1.4 and 0.95 bulbs per explant, respectively, compared with 0.60 bulbs per explant grown on solid medium (Table 5). Liquid shake culture was unsuccessful as no growth occurred for either leaf or shoot explants. Similarly, lily stationary cultures performed better than shake cultures (Varshney et al., 2000). This may have been a result of shearing or possibly leaching of nutrients. However, Takayama et al. (1991) found liquid shake culture to be better for the propagation of shoots and bulbs in *H. orientalis*.

Liquid static culture was not only a useful technique for the production of bulblets but also for the enhancement of bulblet growth and size. Mean bulb diameter and mass was considerably higher when explants were placed in liquid culture compared to those grown on an agar solidified medium. Leaf explants in static liquid culture produced bulbs with a mean diameter of 2.75 mm and a mean mass of 45.38 mg. Bulblets that developed from shoots in static liquid culture were even bigger with a mean diameter and mass of 5.47 mm and 162.57 mg. Bulblets that formed on leaf explants on solid MS were considerably smaller with an average diameter of 2.42 mm and mean mass of 23.40 mg. Liquid culture can thus be used to improve bulblet growth. This was reported by Morán et al. (2003) who found that liquid culture with 30 or 60 g/L sucrose can be used to accelerate biomass gain and the bulbing of small-sized shoots for both *Cyrtanthus clavatus* and *Cyrtanthus spiralis*. It could therefore be beneficial to place bulbs in liquid culture for a couple of weeks to increase bulb growth prior to transplanting to soil. This will increase the survival rate of bulblets *ex vitro*. This method could be useful for the commercialization of *E. zambesiaca* plants but requires further investigation.

All the *in vitro* grown bulblets that were placed in the misthouse grew in size and formed leaves and roots. After removal from the misthouse and transplantation, some of the bulbs lost their leaves and did not survive. Overall, there was an 80–90% survival rate.

This study provides a useful protocol to ensure the production of *E. zambesiaca* bulbs that could be used to supply plants to meet the medicinal and horticultural needs and prevent large scale collection from the wild.

Table 5
Effect of different liquid culture treatments on bulblet induction on leaf explants and shoots of *E. zambesiaca*.

Treatment	Explant part	No. of bulblets per explant	Bulblet diameter (mm)	Bulblet mass (mg)
Solid	Leaves	0.60 ± 0.22^{c}	2.42 ± 0.41^{b}	23.40 ± 6.92^{c}
Liquid shake	Leaves	0.00 ± 0.00^{d}	0.00 ± 0.00^{c}	0.00 ± 0.00^{d}
Liquid shake	Shoots	0.00 ± 0.00^{d}	0.00 ± 0.00^{c}	0.00 ± 0.00^{d}
Liquid static	Leaves	1.40 ± 0.20^{a}	2.75 ± 0.10^{b}	45.38 ± 5.26^{b}
Liquid static	Shoots	0.95 ± 0.07^{b}	5.47 ± 0.52^{a}	162.57 ± 25.40^{a}

Mean values in the same column followed by different letters are significantly different ($p \leq 0.05$).

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