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Developing an *In Vitro* Propagation Method for Mass Production of Medicinal Hypoxis Species Using Bioreactors

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ABSTRACT

Hypoxis species are amongst the most important medicinal plants of southern Africa in traditional medicine, and in the composition of pharmaceutical products. These perennial grasses belong to the Hypoxidaceae family and occur naturally. Bioreactors are suitable for vegetative propagation of plant materials for mass production, but have not been previously reported in Hypoxis species. In this study micropropagation of Hypoxis species using bioreactors was evaluated for future mass production using two species: H. argentea Harv. ex Baker var. argentea and H. filiformis Baker. Corms of both H. argentea and H. filiformis and shoots of H. filiformis, initially established on semi-solid medium were used as explants. Explants were cultured for 10 weeks in bioreactors containing the MS medium supplemented with different types and concentrations of plant growth regulators. The results showed that both corm and shoot cultures can be used as explants for shoot regeneration and multiplication of both species. The most suitable medium for achieving better proliferation differed between the two species. For H. filiformis, the highest regeneration rate of 85% with the highest multiplication rate of 12 was achieved with corm explants cultured in the PGR-free MS. For H. argentea the highest regeneration rate of 72%, from corm explants was also obtained from the same medium, but the highest multiplication rate of 5 was obtained from the MS medium supplemented with 3 mg/l kinetin. Shoot cultures of H. filiformis achieved 75% regeneration rate in the medium supplemented with 1.1 mg/l TDZ. Establishment of acclimatized shoots ex vitro was 86%.

Keywords: Bioreactor, *Hypoxis*, Micropropagation, Plant growth regulators, Temporary immersion

Abbreviations: BA: Benzyl Adenine; PGR(s): Plant growth regulator(s); TDZ: Thidiuzuron

INTRODUCTION

The use of traditional medicine plays a significant role in the health and welfare of people in developing countries [1]. Most of the phytomedicines that are used in these countries are prepared from plant parts of wild species; thus it is feared that a large number of wild medicinal plant species will become extinct due to constant harvest without replacement, which is no longer sustainable [2]. There is a growing need to develop strategies for commercializing the production of medicinal plants. Propagation of these species by conventional methods has encountered several problems such as insufficient seed stocks, and harvesting the seeds from the wild populations will only accelerate the problem of diminishing stocks, as well as decrease the gene pool [3].

Species of Hypoxis, belonging to the Hypoxidacea family, are amongst the most important medicinal plants of southern Africa and are popular in traditional medicine for treating many ailments [4-6] as well as in the composition of some pharmaceutical products. *Hypoxis hemerocalidea*, African potato, has made a significant mark in the pharmaceutical industry resulting to its extensive and unsustainable harvesting from the wild, and placing it at risk of extinction. To prevent this and to effectively preserve the wild materials, efforts of commercially producing this species and others of medicinal value, have been made [7]. Hypoxis species are not easily propagated by conventional vegetative methods mainly because vegetative propagation from corms is difficult, and the seeds have a relatively strong dormancy that is not easy to break [8-10]. Micropropation has shown to be a good alternative for vegetative propagation of Hypoxis. Positive findings have been made in the regeneration of Hypoxis species via organogenesis using conventional *in vitro* methods with semi-solid medium, but very little progress has been made towards commercial use of these methods

for their mass production. So far there is no report on efforts to massively propagate Hypoxis using bioreactors for shoot regeneration.

Conventional micropropagation on semi-solid media for mass production is labour intensive and costly [11], while the use of bioreactors with the temporary immersion principle is more cost-effective, especially for rare and slow growing species [12]. Bioreactors provide a fast and efficient plant propagation system for many agricultural and forestry species with liquid medium to reduce manual handling [13] and production costs. Some progress in the use of bioreactors as a possible means of cost reduction in micropropagation has been made [14]. They have been successfully used in plant regeneration using organs such as shoots, bulbs, microtubers, corms and embryos [15]; and have been tested in the production of horticultural and medicinal plants such as apple [16], Anoectophilus, Chrysanthemum, Lilium, Phalaenopsis, garlic, ginseng, grape and potato [14], Digitalis, Echinacea and Rubus [17]. Alister et al. [18] reported a four to six times increase in yield, in half the time of eucalyptus clones when the cultures grew in the bioreactor system compared to the semi-solid media. The multiplication rate of pineapple shoots (*Ananas comosus* L.) as reported by Escalona et al. [19], increased by up to 400% when using the bioreactor system with temporary immersion compared to that obtained from continuous culture in liquid medium or culture on conventional semi-solid medium.

The studies mentioned above indicate that bioreactors could be used to vegetatively propagate Hypoxis species, as well as scale up and speed up the process of production of the species. The objective of this study was therefore to evaluate the potential of using the temporary immersion bioreactor system for *in vitro* propagation of Hypoxis for large scale production.

MATERIALS AND METHODS

The study was conducted at the Swedish University of Agricultural Sciences (SLU), Department of Plant Breeding, Alnarp, Sweden. Two species of Hypoxis were used, *Hypoxis argentea* Harv. ex Baker var. argentea and *Hypoxis filiformis* Baker that had been previously established *in vitro* on semi-solid medium. Cormlets (Figure 1A) and young shoots from the *in vitro* cultures of *H. filiformis* were used as explants whilst only cormlet explants for *H. argentea* were tested. For corm cultures, the shoots were removed from the cormlets before culturing, for the shoot cultures, shoots were cut just below the base of the corms ensuring that a small part of the corm remained attached to the shoots.

Explants were cultured in the liquid MS [20] medium containing 30 g/l sucrose and 1 g/l calcium hydrolysate with pH 5.8. The medium was supplemented with PGRs of different types and concentrations. Corm explants were cultured in either the PGR-free MS medium or MS medium supplemented with PGRs as follows: i) 3 mg/l benzyl adenine (BA), ii) 3 mg/l kinetin, iii) 1.1 mg/l thidiazuron (TDZ). Shoot explants were also cultured in either the PGR-free MS medium or MS medium supplemented with PGRs as follows: i) 3 mg/l BA, ii) 3 mg/l kinetin, iii) 0.55 mg/l TDZ, iv) 1.1 mg/l TDZ, v) 2.2 mg/l TDZ. The explants were cultured in the same type of medium for 10 weeks and transferred into fresh medium once.

The PlantForm® bioreactors (Figure 1B) using the temporary immersion principle as described by Persson [21], were used for culturing the explants. The explants were immersed in the media twice a day for the duration of 6 min per immersion and 8 h apart. Aeration was provided every hour during the day for the duration of 4 min for 8 h. All cultures were grown under the same climate conditions: temperature of 25/18°C (day/night) and 16 h photoperiod and the light intensity of 40 µmol m^{-2} s^{-1} provided by cool white fluorescent tubes. Each treatment was repeated 2 or 3 times depending on the availability of materials. Sixteen explants per replicate were used for the corm cultures and 20 for the shoot cultures. Data were statistically analyzed using Minitab 16.

Regenerated shoots from the bioreactors were acclimatized using the protocol described by Nsibande et al. [10] before being grown in the greenhouse. Twenty-four 14 week old plantlets were planted in the field..

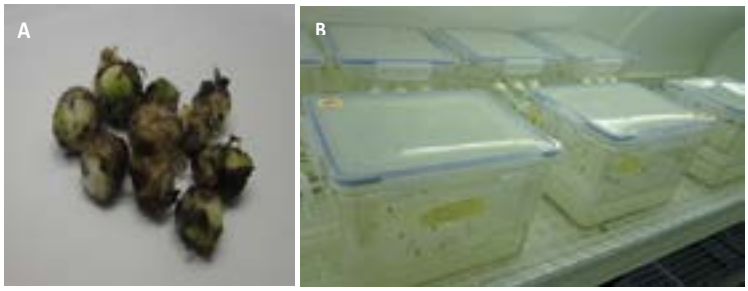

Figure 1: *In vitro* **co**rmlets used as explants (a) and interconnected bioreactors used in this study (b)

RESULTS AND DISCUSSION

Corm explants of *H. filiformis*

Significant differences were observed in percentages of explants that produced shoots ($p<0.01$), callus ($p<0.05$) or roots ($p<0.001$) from the corm explants of *H. filiformis* in the different media (Figure 2), as well as in the number of shoots obtained from each regenerated explant ($p<0.001$). The explants cultured in the PGR-free MS (Figure 3A) yielded the highest shoot regeneration rate of 85.4%, followed by those cultured in the MS medium supplemented with 3 mg/l kinetin, with 60.4% (Table 1). The highest shoot number of 12 per regenerated explant was also achieved from the PGR-free MS, this was followed by 6 obtained from the medium supplemented with 1.1 mg/l TDZ (Figure 4). The highest rooting percentages were obtained from the medium supplemented with 3 mg/l kinetin (29.2%) and the PGR-free MS (27.1%). MS medium supplemented with 1.1 mg/l TDZ resulted in the highest percentage (83.1%) of explants that formed calli.

Table 1: Explants with shoots and no. of shoots/explant obtained from corm explants of *H. argentea* and *H. filiformis* cultured in different media

Substrate	Species	Explants with shoots (%)	No of shoots/explant
PGR free MS	*H. argentea*	71.9 ± 21.9	3 ± 0.51
	H. filiformis	85.4 ± 7.51	12 ± 1.0
3 mg l^{-1} BAP	*H. argentea*	59.4 ± 21.9	3 ± 0.59
	H. filiformis	41.7 ± 9.54	4 ± 0.79
3 mg l^{-1} Kinetin	*H. argentea*	62.5 ± 12.5	5 ± 0.84
	H. filiformis	60.4 ± 2.07	2 ± 0.65
1 mg l^{-1} TDZ	*H. argentea*	-	-
	H. filiformis	25.0 ± 3.58	6 ± 1.26

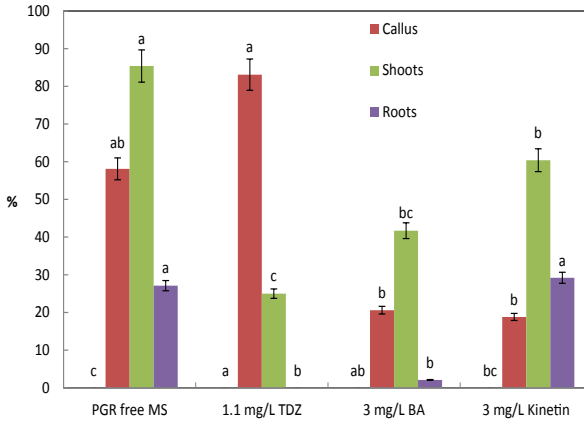

Figure 2: Percentage of corm explants of *H. filiformis* showing callus, shoot, and root formation when cultured in the different media for 10 weeks. Bars with different letters for each parameter indicate significant differences among the different media

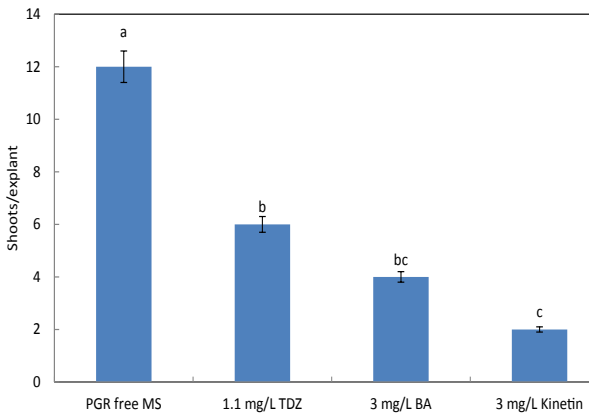

Figure 3: Shoot number per regenerated corm explant of *H. filiformis* cultured in the different media for 10 weeks. Bars with different letters indicate significant differences

Corm explants of *H. argentea*

In the three types of media in which the corm explants of *H. argentea* were cultured, no significant differences ($p>0.05$) were observed in the percentages of explants with shoots, callus or roots for this species. Significant differences ($p<0.05$) were, however observed in the shoot number per regenerated explant where the highest shoot number 5 was obtained from the MS medium supplemented with 3 mg/l kinetin (Figure 5). The shoot regeneration rate ranged between 59.4% and 71.9% (Table 1), with the PGR-free medium giving the seemingly higher value which, however, was not significantly different from that obtained from the MS medium supplemented with either 3 mg/l kinetin (Figure 3B) or with 3 mg/l BA. No significant differences in root formation were found between the PGR-free MS and the media supplemented with PGRs.

Further comparison showed that the shoot regeneration rate of corm explants between the two species cultured in the same type of media did not significantly differ. However, results of the shoot number were significantly different ($p<0.05$) in that the number of shoots per regenerated explant (12) obtained from corm explants of *H. filiformis* cultured in the PGR-free MS was significantly higher (3) than that of *H. argentea* in the same medium (Figure 6). Corm explants of both *H. argentea* and *H. filiformis* produced roots, to some extent, in all the media with the exception of *H. filiformis*, which did not root when cultured in the medium containing 1.1 mg/l TDZ.

Shoot explants of *H. filiformis*

Highly significant differences ($p<0.01$) were obtained from explants of *H. filiformis* that produced callus, shoots and roots in the different media (Figure 7). In the MS medium supplemented with 1.1 mg/l TDZ shoot cultures produced 85% callus, which was significantly higher than that produced in the PGR-free MS, or the MS supplemented with any of the other PGRs. The highest shoot regeneration rate (75%) was also obtained from the medium supplemented with 1.1 mg/l TDZ (Table 2), while the medium supplemented with 0.55 mg/l TDZ produced almost the same regeneration rate (72.5%) (Figure 3C). There was no significant difference observed in shoot number per regenerated explant among the different types of media. The shoot explants had significantly more explants producing roots (45%) when cultured in the MS supplemented with 0.55 mg/l TDZ, followed by those cultured in the MS supplemented with 1.1 mg/l TDZ or 2.2 mg/l TDZ (27.5%). No roots were produced by the explants that were cultured in the MS supplemented with 2 mg/l BA or the MS supplemented with 3 mg/l BA. However, when unrooted shoots were cultured in PGR-free MS for 10 weeks, 40% of rooting was achieved.

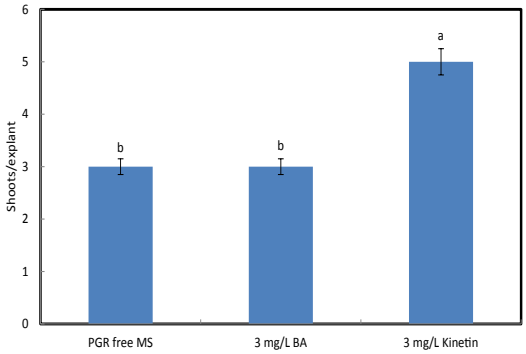

Figure 4: Shoot number per regenerated corm explant of *H. argentea* cultured in the different media for 10 weeks. Bars with different letters indicate significant differences

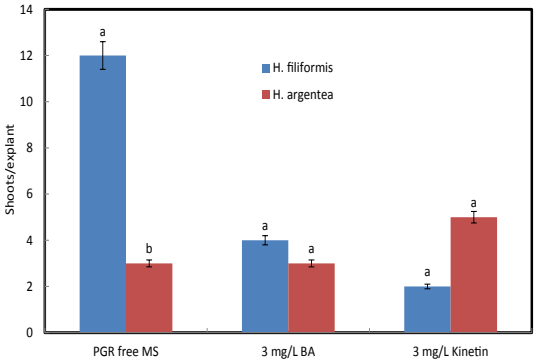

Figure 5: Comparison of the shoot number per regenerated corm explants between *H. filiformis* and *H. argentea* cultured in the different media for 10 weeks. Bars with different letters indicate significant differences between the two species cultured in the same type of medium

Table 2: Explants with shoots and no. of shoots/explant obtained from shoot explants of *H. filiformis* cultured in different media

Substrate	Explants with shoots (%)	No. of shoots/explant
PGR free MS	27.5 ± 22.5	1 ± 0.12
0.55 mg l^{-1} TDZ	72.5 ± 7.5	3 ± 0.65
1.1 mg l^{-1} TDZ	75.0 ± 15.0	4 ± 0.60
2.2 mg l^{-1} TDZ	50.0 ± 0	3 ± 0.54
2 mg l^{-1} BAP	50.0 ± 10.0	3 ± 0.77
3 mg l^{-1} BAP	62.5 ± 32.5	4 ± 0.62

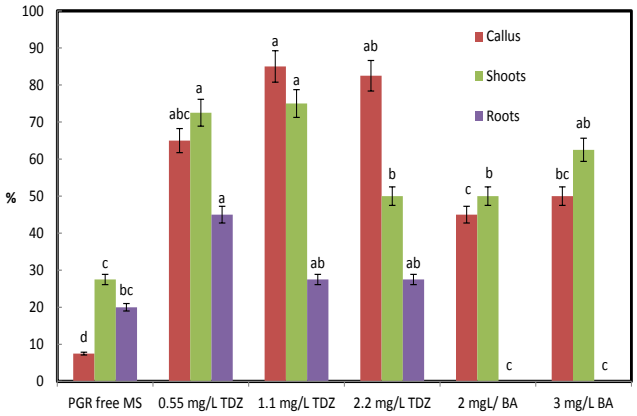

Figure 6: Percentage of shoot explants of *H. filiformis* showing callus, shoot and root formation when cultured in the different media for 10 weeks. Bars with different letters for each parameter indicate significant differences among the different media

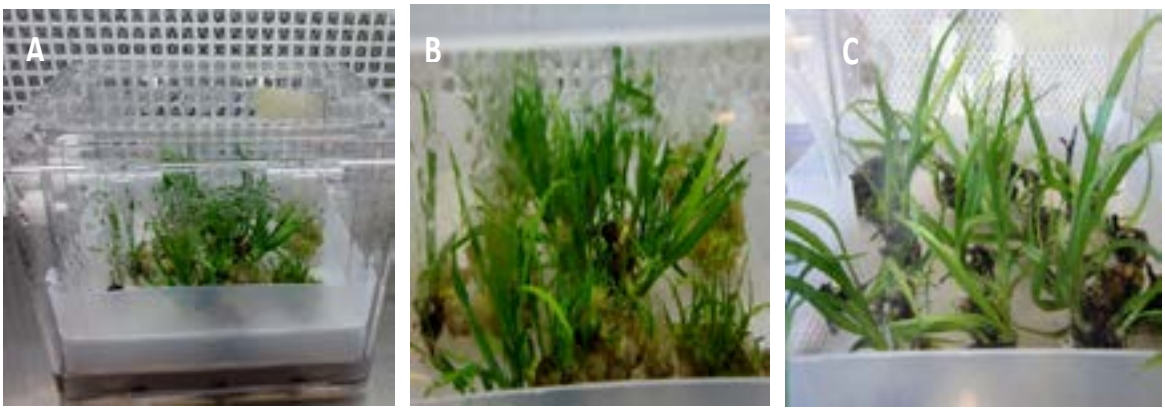

Figure 7: Images of cultures of *H. filiformis* and *H. argentea* in the different media**; A** 10 week old corm cultures of *H. filiformis* in PGR-free MS; **B** 10 week old corm culture of *H. argentea* in 3 mg l^{-1} kinetin; **C** 10 week old shoot cultures of *H. filiformis* in 0.55 mg l^{-1} TDZ

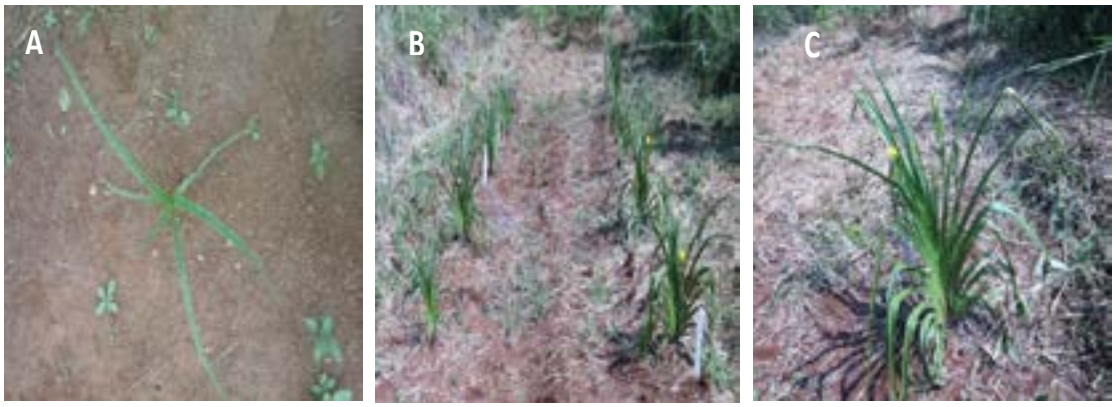

Figure 8: Established plants of *H. filiformis* and *H. argentea* in the field genebank at the Malkerns Research Station, Swaziland; a) Young *Hypoxis* plantlet from the bioreactor established in greenhouse for 14 weeks; b) *H. argentea* and *H. filiformis* from semi-solid medium established in greenhouse for 9 months; c) *H. argentea* from semi-solid medium established in greenhouse for 14 weeks

Greenhouse establishment

Eighty-six percent of the acclimatized shoots regenerated from the corm and shoot explants of *H. argentea* and *H. filiformis* were successfully established in the greenhouse. Efforts to establish some of the plants that had been in the greenhouse for 14 weeks in the field were not very successful, most likely due to the dry and hot weather conditions that prevailed during the planting season combined with the physiological status of the plants as the corms were very small during the transplant, and may not have assumed the capacity to sustain the plants. Out of 24 plantlets planted only 3 survived (Figure 8A). Plantlets that were kept in the greenhouse for 9 months after successful *in vitro* establishment from semi-solid medium survived better (Figures 8B and 8C).

DISCUSSION

A few studies on using bioreactors for mass production of different plant organs and products from plant materials have been reported in several plant species, such as the production of podophyllotoxin from *Podophyllum hexandum* [22], mass multiplication of protom-like bodies in Phalaenopsis [23], mass production of somatic embryos in *Coffea arabica* [24], mass production of pineapple plants [19], production of Eucalyptus clones [18].

In this study we have, for the first time, evaluated the potential of bioreactor application for mass production of medicinal Hypoxis and have achieved positive outcomes. Our results showed that as start materials corm explants behave differently from shoot explants even though both can be used for bioreactor production. Corm explants responded faster to *in vitro* conditions in the bioreactors than shoot explants, as within a week of culturing a green pigment indicating shoot growth could be observed. On the other hand, shoot cultures started by seemingly dying off before new shoots were formed; part of the shoot or the whole shoot would turn brown giving the impression that it was dead. The highest shoot regeneration rate and the highest multiplication rate were both obtained from the corm explants of *H. filiformis*.

The results from this study showed a species-dependant response to the same *in vitro* culture conditions. For instance, corm explants of the two species *H. filiformis* and *H. argentea* responded differently to different media under the same culture conditions. *H. filiformis* seemed to respond faster and better compared to *H. argentea*, the same observation was made by Nsibande et al. [10] when these species together with two others (*H. acuminata* and *H. hemerocallidea*) were cultured on semi-solid medium using the conventional *in vitro* method. In the same study, Nsibande et al. [10] obtained 100% regeneration and a multiplication rate of 17 shoots from corm explants of *H. filiformis* cultured on the semi-solid MS medium supplemented with 3 mg/l kinetin for 24 weeks. In this study the highest regeneration rate and the highest multiplication rate obtained from corm explants of *H. filiformis* were 85% and 12, respectively from the PGR-free MS within 10 weeks. Within the same period, the highest regeneration rate and multiplication rate of 62.5% and 5, respectively, were obtained from corm explants of *H. argentea* that were cultured in the MS medium supplemented with 3 mg/l kinetin. However, when corm explants of this species were cultured on semi-solid medium containing the same type and concentration of PGR for 24 weeks, Nsibande et al [10] obtained a shoot regeneration rate of only 22.2% and a multiplication rate of 3. This implies that by using bioreactors more shoots can be generated over a relatively shorter period compared to semi-solid medium and that the genotype dependancy of Hypoxis species to *in vitro* conditions exists. Also worth noting is that corm explants of *H. filiformis* performed better when cultured in the PGR-free medium as the multiplication rate (12) was significantly higher than the highest multiplication rate (6) obtained from medium supplemented with PGRs. The opposite tendency was observed with *H. argentea* as the highest multiplication rate (5) was obtained from the PGR-supplemented medium and the lowest (3) from the medium without PGRs. This suggests that with corm explants of *H. filiformis*, once organogenesis has been initiated *in vitro*, it is no longer necessary to further supplement preceding explants with PGRs, specifically cytokinins. It is most likely that with this species the endogenous cytokinins present in the explants are enough to sustain shoot development, at least for the first few weeks, whereas those of *H. argentea* would still require cytokinins for shoot proliferation. Nsibande et al [10] have shown that corm explants of both *H. filiformis* and *H. argentea* cultured on semi-solid medium, directly from *ex vitro* material produced the highest shoot regeneration and multiplication rates from media supplemented with cytokinins. Similar results have also been reported by Appleton and van Staden [8,25] and Ndong et al. [9] on corm cultures of some Hypoxis species on semi-solid medium. However, in the case of shoot explants of *H. filififormis*, addition of cytokinins to medium is still necessary for shoot organogenesis irrespective of whether the explants used are derived from *in vitro* material that had been cultured with addition of cytokinins or not, suggesting that the endogenous cytokinins present in the shoot explants are insufficient for shoot regeneration of these explants. Moreover, based on visual observation, direct organogenesis from corm explants was more common than de novo organognesis via callus in both species but less so in shoot explants of *H. filiformis*.

CONCLUSION

This work has shown that shoot regeneration of Hypoxis can be induced with high shoot multiplication rate within a relatively short period of time using the temporary immersion bioreactor system. Both corms and shoots can be used as explants, but the corms provide high productivity. Even though the two species, *H. filiformis* and *H. argentea*, responded differently to the same *in vitro* conditions they both showed positive results in shoot production. We can thus conclude that it is possible to use the bioreactor system for mass production of Hypoxis for commercial purposes provided the culture conditions for each species are optimized. This platform allows for a large number of plant materials to be cultured at the same time within a limited area under optimal conditions, thus making it more cost-effective compared to conventional *in vitro* cultivation techniques.

ETHICAL APPROVAL

The authors wish to ascertain that this manuscript has not been submitted to any other journal for publication and have adhered to all ethical considerations.

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