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In vitro regeneration of *Hypoxis colchicifolia* plantlets

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Abstract

Corms of *Hypoxis* species are heavily traded for use in traditional medicine in southern Africa. High demand has increased unsustainable harvesting from the wild, diminishing natural populations. Micropropagation of *Hypoxis colchicifolia* was investigated as a means of mass producing plants for both commercialization and re-establishment in the wild. Various plant organs were tested as explant sources and decontamination optimized for each explant type. Seeds failed to germinate *in vitro*, and inflorescence peduncles, leaves (young and mature) turned brown and did not respond in culture. Only 6% of corm explants produced callus and shoots. Flower buds responded best, with multiple shoots initiated from explants either directly from meristemoids or indirectly via callus. The problem of browning due to phenolic exudation was solved by including polyvinylpyrrolidone (PVP) in the culture medium when required. Common pathogens were partially controlled by washing affected explants in benomyl solutions. Rooting and corm induction were successful, and plantlets could be stored at low temperature (10 °C) prior to acclimatization with no adverse effects. In planting trials with 5-month-old and 21-month-old plants regenerated using the improved protocols, flowering percentage, corm and leaf size were increased significantly in plants grown in pots compared to those grown in the field over a 28 month period.

Keywords: Decontamination; *Hypoxis*; Medicinal plant; Micropropagation; South Africa; Tissue culture

1. Introduction

In southern Africa, traditional medicines prepared from *Hypoxis* plants (Family Hypoxidaceae) are variously used as tonics, purgatives and diuretics, or to treat infertility, inflammation, prostate gland disorders, wounds and burns. Not as well documented is their widespread use in traditional veterinary medicine to treat gall-sickness, infertility, heart-water, sores and cracked hooves of livestock (Van der Merwe, 2000; Watt and Breyer-Brandwijk, 1962). While eleven species of the genus *Hypoxis* are used for traditional purposes throughout southern Africa, as summarised by Appleton (2004), surveys of the most popular medicinal plants traded in South Africa have identified only two species, *H. hemerocallidea* and *H. colchicifolia*, as routinely sold at medicinal plant markets in eastern KwaZulu-Natal (Cunningham, 1988; Von Ahlefeldt et al., 2003) or via mail-order from herbal traders in the Gauteng Province (Williams, 1992). Surveys conducted in the Eastern Cape Province have also shown *H. hemerocallidea* to not only be the most frequently traded medicinal plant (Dold and Cocks, 2002), but also, together with *H. colchicifolia*, they rank among the four plant species most sought after for use in the traditional treatment of diabetes (Mahop and Mayet, 2007).

Traditional African medicine has contributed significantly to the commercial status of the genus. Compounds of value found in the corms of the most studied species, *H. hemerocallidea*, popularly known as the 'African Potato', are the normal phytosterols, in particular beta-sitosterol and its glycoside, and the phenolic glycoside, hypoxoside. Therapeutic products developed from dried corm powder contain standardised amounts of phytosterols and sitosterolins specifically formulated to either treat benign prostatic hypertrophy (Pegel, 1984) or modulate the human immune system (Bouic et al., 1996; Drewes et al., 2006;

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Vanderhaeghe and Bouic, 1999). Albrecht (1996) proposed that hypoxoside might be effective as an oral, non-toxic pro-drug in the treatment of inflammation, HIV-infection and cancer therapy as it is readily converted to the more active anti-cancer compound rooperol. Claims such as these resulted in the development of a range of *Hypoxis* extracts, tinctures and creams for the popular herbal industry in South Africa. To meet this new demand a single manufacturer is known to produce several tons of 'Impilo African Potato' per month (Drewes et al., 2006). Other studies to determine the efficacy of several medicinal plants used in traditional remedies have shown *H. colchicifolia* (= *H. latifolia*) to be amongst four of the plants tested to exhibit the best antibacterial activity (Buwa and Van Staden, 2006), while extracts of *H. colchicifolia* leaves displayed the best anthelmintic, antibacterial and antifungal activity (Aremu et al., 2010).

Kruger et al. (1994) also emphasised the importance of selecting the correct *Hypoxis* species for possible therapeutic application when *H. colchicifolia* corm extracts were shown to contain a higher proportion of rooperol than those of *H. hemerocallidea*. A serious constraint encountered during the development of plant-derived drugs, however, is the difficulty to source sufficient correctly identified plants for evaluation. To address this need and clarify the nomenclature of *Hypoxis* a list of African *Hypoxis* species with their accepted names has been presented and includes *H. hemerocallidea* and *H. colchicifolia* (Singh, 2006). The validly published names *H. rooperii* and *H. latifolia*, among others, however, have been reduced to synonyms of *H. hemerocallidea* and *H. colchicifolia* respectively (Burtt, 1986; Singh, 2007).

Hypoxis plants are generally not cultivated due to the perception that they are naturally abundant, a viewpoint shown to be invalid for traditionally high usage areas in the province of KwaZulu-Natal where popular species such as *H. hemerocallidea* may be locally extinct (Scott-Shaw, 1999). Wild *Hypoxis* plants are, therefore, destructively harvested to meet demand. The pressure of collection on the natural populations of *Hypoxis* is not easily quantified, however, since few ecological or market studies have been undertaken. Geophytes are also poor indicators of demand and supply as their underground organs are totally removed during harvesting leaving no trace of previous plant density. This was confirmed in a study which showed that within seven months of commencement all available *H. hemerocallidea* and *H. colchicifolia* plants had been removed from the traditional collecting sites monitored (Naidoo, 1998). In addition, data gathered on the density of *H. hemerocallidea* and *H. colchicifolia* plants in their natural habitats showed that human activities such as manual grass cutting and partial management of open spaces, and the grassland management practices of mowing and burning resulted in a 75% reduction in plant density of these species. Mature *H. colchicifolia* plants were also totally destroyed in frequently mown and burnt areas and those undergoing urban development (Appleton, 2004). With respect to early market surveys, high sales figures for *H. hemerocallidea* and *H. colchicifolia* corms were recorded at the time. It was calculated that approximately 31,300 corms of each species were sold annually from only 54 outlets in Durban (Cunningham, 1988), while 11,000 kg of *H. hemerocallidea* corms were sold in the Eastern Cape Province (Dold and Cocks, 2002). These figures support the argument that *Hypoxis* plants are harvested unsustainably from natural habitats and should be cultivated to ensure their survival, conserve natural populations and supply various industries.

Translocation of rescued plants has been suggested as a means to both save and propagate *Hypoxis*. While plants of several *Hypoxis* species were successfully relocated from a condemned area to new sites, seed collected from *H. acuminata* plants thereafter failed to germinate (Kroon, 1999). Natural regeneration is reliant on germination from viable seed and would be essential for translocation to succeed as *Hypoxis* plants rarely multiply vegetatively. Natural seedling recruitment, however, was found to be insufficient to sustain wild populations of either *H. hemerocallidea* or *H. colchicifolia* when subjected to the pressures of collection (Naidoo, 1998; Nomtshongwana, 1995). Although *H. hemerocallidea* seedlings have been obtained (Gillmer and Symmonds, 1999), propagation of sufficient plants from seed to meet demand has proven difficult due to their deep dormancy, inconsistent germination, and variable viability both within a season (Hammerton et al., 1989) or seasonally from year to year (Nomtshongwana, 1995).

Micropropagation was, therefore, examined as an alternative method to propagate *Hypoxis*. Initially, techniques to produce *H. hemerocallidea* (= *H. rooperii*) plants *in vitro* from corms and flower buds were developed (Page and Van Staden, 1984, 1986). All the *H. hemerocallidea* plants used in an extensive field trial thereafter (McAlister and Van Staden, 1995) were produced *in vitro* as per Page and Van Staden (1984) and Van Staden and Bayley (1988). The establishment of these mature cloned plants in cultivation clearly demonstrated that *H. hemerocallidea* plants can be successfully propagated *in vitro* for agricultural and conservation purposes. Other *Hypoxis* species have responded differently to *in vitro* culture, however, particularly with respect to plant growth regulator requirements. This was demonstrated when cultures of *H. obtusa* were obtained, but explants of *H. nyasica* and *H. angustifolia* failed to regenerate under the same conditions (Vinesi et al., 1990). To address the need for a standard *in vitro* protocol suitable for the mass propagation of all the medicinal species of *Hypoxis*, the original techniques reported for *H. hemerocallidea* (Page and Van Staden, 1984, 1986) were modified by introducing distinct sequential steps into the procedure (Appleton and Van Staden, 1995a). Continuous plantlet regeneration was obtained for *H. hemerocallidea*, *H. acuminata*, *H. rigidula* and *H. obtusa* using the amended protocol. The primary corm explants of *H. colchicifolia* responded poorly to *in vitro* culture, however, due to the negative and toxic effects of browning and internal pathogens. Although slight differences in plant growth regulator requirements were again reported, the stepwise *in vitro* protocol was also successful in the regeneration of plantlets of the non-medicinal species, *H. angustifolia* var. *angustifolia*, via both direct and indirect organogenesis (Appleton and Van Staden, 1995b). Similar results to the above were obtained while developing *in vitro* protocols for *H. hemerocallidea* landrace Gaza from Mozambique (Ndong et al., 2006). It should be noted, however, that *H. rooperii* is a synonym of