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BRIEF COMMUNICATION

Micropropagation of *Harpagophytum procumbens*

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Abstract

An efficient protocol for micropropagation of *Harpagophytum procumbens* DC., an endangered African medicinal plant, was developed. Maximum shoot multiplication without callus was obtained from nodal explants cultured on Murashige and Skoog (MS) basal salts plus Gamborg's (B_5) vitamins supplemented with 0.1 mg dm^{-3} indole-3-acetic acid and 5.0 mg dm^{-3} kinetin. The shoots were subsequently subcultured every 3 weeks on the same medium. Detached axillary shoots were transferred to MS basal salts plus B_5 vitamins supplemented with various concentrations of α-naphthalene-acetic acid or indole-3-butyric acid (IBA), ranging from 0.5 to 2.5 mg dm^{-3} and 100 % rooting and optimal subsequent acclimatization was achieved on 1.0 mg dm^{-3} IBA. After 4 weeks of culture, the rooted shoots (>5 cm) were planted in pots containing peat, vermiculite and bark (2:1:1), covered with plastic domes and maintained at 25 °C for 2 weeks before being transferred to a glasshouse. Plant survival was about 40 %.

Additional key words: acclimatization, auxins, cytokinins, devil's claw, grapple plant, tissue culture.

The genus *Harpagophytum* (*Pedaliaceae*), comprising two species, *H. procumbens* (Burch.) DC. *ex* Meissner and *H. zeyheri* Decne, occurs in Namibia, Botswana, South Africa, Zambia, Zimbabwe and Mozambique. The species, commonly called devil's claw or grapple plant, is a perennial that is indigenous to southern Africa. Due to its high medicinal importance (Van Haelen *et al*. 1983), rural communities intensively harvest the species in the field. Furthermore, the seeds exhibit a high degree of dormancy (Ernst *et al*. 1988) resulting in very poor seedling recruitment. Plant tissue culture techniques provide a suitable alternative method for mass propagation of this endangered species (Levieille and Wilson 2000, 2002, Afolayan and Adibola 2004). The present study was initiated with two-fold objectives: *1*) to develop a suitable method to improve the seed germination percentage, and *2*) to formulate a protocol for the rapid micropropagation of *Harpagophytum procumbens.*

Seeds from dry fruits and primary tubers of *Harpagophytum procumbens* DC were collected in May 2000 from the field in Kuruman, Northern Cape, South Africa. The nodal and shoot tips explants for the micropropagation experiments were obtained from *in vitro* developed seedlings or young actively growing shoots developed from tubers planted in pots containing two parts of potting soil and one part of river sand and maintained in an air-conditioned glasshouse set at 25 °C with a relative humidity of 70 %, under natural light. To obtain seedlings *in vitro*, the dormant seeds, after surface sterilization in 0.5 % (m/v) sodium hypochlorite for 1 min, followed by 3 washes in distilled water, were subjected to a series of pre-treatments such as alternating temperatures, acid treatment, scarification, pricking of seeds at the basal end and subsequent soaking in distilled water and gibberellic acid (GA_3) with concentrations ranging from 100 to 300 mg dm^{-3}. Out of these pre-treatments tried, the seeds, which were pricked at the

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Abbreviations: ADE - adenine; AH - adenine hemisulphate; BAP - 6-benzylaminopurine; GA_3 - gibberellic acid; IAA - indole-3-acetic acid; IBA - indole-3-butyric acid; Kn - kinetin; MS - Murashige and Skoog; NAA - α-naphthalene acetic acid; PGR - plant growth regulator; Z - zeatin.

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basal end and soaked in GA_3 overnight responded well. Ernst *et al*. (1988) also reported the positive influence of GA_3 for seed germination of two *Harpagophytum* species. The soaked seeds were then surface-disinfected with 0.1 % $HgCl_2$ (m/v) for 3 min, followed by 3 washes in sterile distilled water, and transferred to half-strength MS basal medium (Murashige and Skoog 1962) containing 1 % (m/v) sucrose and 0.8 % (m/v) agar. The seeds were then incubated in dark at 25 ± 1 °C until the emergence of the radicle. The germinated seeds were then transferred to a 16-h photoperiod. Six-week old, aseptically grown seedlings were then used as the source of explants. For culture initiation, the stem segments (5 - 10 mm) with two to three nodes each, and shoot tips (10 - 15 mm) obtained from the primary tuber derived young actively growing shoots, were surface-disinfested as described above and transferred to medium containing MS basal salts with Gamborg's (B_5) vitamins (Gamborg *et al*. 1968), 3 % (m/v) sucrose, 0.1 mg dm^{-3} indole-3-acetic acid (IAA) and 5.0 mg dm^{-3} kinetin (Kn). Similarly, the explants obtained from *in vitro* developed seedlings were inoculated to the above medium. The pH of the medium was adjusted to 5.8 before sterilizing by autoclaving at 121 °C for 15 min. The primary explants were cultured for 4 to 6 weeks and subsequent subculturing was performed on the same medium.

For shoot multiplication, stem segments, approximately 10 mm in length (one node), were excised from the *in vitro* established clones and transferred to MS medium with Gamborg's (B_5) vitamins supplemented with various combinations and concentrations of naphthalene acetic acid (NAA), 6-benzylaminopurine (BAP), IAA, Kn, adenine (ADE), adenine hemisulphate (AH) and zeatin (Table 1). After four weeks in culture, the efficiency of each media combinations on shoot proliferation and growth was determined by recording the number and length of axillary shoots per explant, percentage of roots and callus formation, if any, at the basal portion of the shoot. Treatments were carried out with 20 explants and each treatment was repeated twice.

In order to induce rooting, 1-month-old shoots (>5 mm) were transferred to half-strength MS basal medium with Gamborg's (B_5) vitamins supplemented with indole butyric acid (IBA) or NAA at various concentrations ranging from 0.5 to 2.5 mg dm^{-3}, followed by 2 to 3 weeks culture on phytohormone free, half-strength MS medium. After 4 weeks of culture, the number of roots, root length, rooting percentage with or without callus were recorded. The experiment was repeated twice with 20 explants each.

For the initial establishment of clones, cultures were grown in *Magenta GA-7-3* vessels (*Sigma-Aldrich*, South Africa) and for subsequent treatments, cultures were grown in 150 × 25 mm culture tubes with 10 cm^3 culture medium each. All cultures were grown in a growth chamber under temperature of 25 ± 1 °C and 16-h photoperiod with photon flux density of 40 μmol m^{-2} s^{-1} provided with 40 W white fluorescent tubes.

Before they were planted in pots containing peat, vermiculite and bark (2:1:1), shoots were rinsed in sterile warm water to remove the culture medium attached to roots. Pots were shielded with plastic domes to maintain the plants at high humidity and incubated at 25 ± 1 °C for 2 weeks. Plants were then transferred to a glasshouse and placed in a mist room for 3 weeks at 20 °C and 100 % relative humidity under natural light. Plants were weaned by gradually decreasing humidity from 100 to 70 % by venting domes daily for about 5 min and then gradually to 1 h before the plants were transferred to 25 °C, 70 % relative humidity and natural light. Percent survival was recorded 4 weeks after the plantlets were removed from tissue culture. All data were subjected to analysis of variance. When F values were significant, means were separated using Tukey-Kramer multiple comparisons test.

To evaluate the effect of auxins and cytokinins on multiple shoot formation, the nodal explants, obtained from the *in vitro* developed shoot cultures, were used. Regardless of the combinations of auxins and cytokinins used, the nodal explants showed axillary bud formation within two weeks. The mean number and the length of axillary shoots increased significantly with increase in the concentrations of NAA and BAP (Table 1). The maximum number and length of axillary shoots per explant was achieved with 0.3 mg dm^{-3} NAA and 3.0 mg dm^{-3} BAP. Though the positive effect of BAP in combination with NAA was observed in the present study, in all the combinations tried, there was callus formation accompanied by spontaneous root development (Table 1). Shushu (2001) reported a similar observation in *Harpagophytum procumbens* over the effect of NAA and BAP on callus formation during axillary shoot induction. Jha *et al*. (2004) also reported the caulogenic potential of BAP during multiple shoot induction in the cotyledonary explants of *Sesbania rostrata*. Similarly, in a different treatment, the synergistic effect of IAA and Kn was tested (Table 1). Like the earlier combinations of NAA and BAP, the number and the length of axillary shoots increased significantly with increase in the concentrations of IAA and Kn (Table 1). However, unlike NAA + BAP, the treatment with IAA + Kn, neither showed callus growth nor spontaneous root development in almost all the combinations tried. Maximum number of axillary shoots with increased length was attained with 0.1 mg dm^{-3} IAA and 5.0 mg dm^{-3} Kn (Table 1). Besides BAP and Kn, the effects of other cytokinins (AH, ADE, Z) were also tested with IAA at 0.1 mg dm^{-3}. All the combinations, except IAA/Kn, showed significant axillary shoot induction over the control. With the exception of Kn, all cytokinins in combination with IAA, not only increased production of axillary shoots, but also induced root or callus formation. Therefore, based on the different combinations of plant growth regulators studied, the present investigation was in agreement with the earlier reports that low concentration of an auxin can positively modify the shoot induction frequency in combination with cytokinins (Sreekumar *et al*. 2000, Martin 2002, 2003, Wotavová-Novotná *et al*. 2007). Nevertheless callus formation during micropropagation is

Table 1. Effects of combinations of phytohormones on the induction of axillary shoots, callus and root formation in the nodal explants of *H. procumbens* after 4 weeks of culture on MS medium. Values represent mean ± SE (n = 20); means followed by different letters within a column are significantly different at $P < 0.05$ using Tukey-Kramer multiple comparisons test.

Phytohormones	Concentrations [mg dm^{-3}]	Axillary shoots number	length [cm]	Callus formation [%]	Rooting [%]
NAA/BAP	0 / 0	1.9 ± 0.17a	3.31 ± 0.23a	-	-
	0 / 0.5	2.5 ± 0.22a	3.65 ± 0.30ab	60	-
	0.1/ 1.0	2.7 ± 0.26ab	3.67 ± 0.31ab	60	40
	0.2/ 2.0	3.7 ± 0.36bc	3.97 ± 0.28ab	80	70
	0.3/ 3.0	3.9 ± 0.35c	4.63 ± 0.37b	100	90
IAA/Kn	0 / 0	2.1 ± 0.17a	3.38 ± 0.27a	-	-
	0.1/ 1.0	2.8 ± 0.24a	4.54 ± 0.46ab	-	-
	0.1/ 2.0	2.7 ± 0.26a	4.32 ± 0.31ab	-	-
	0.1/ 3.0	5.6 ± 0.47b	5.20 ± 0.43b	-	-
	0.1/ 4.0	6.1 ± 0.64b	5.11 ± 0.45b	-	-
	0.1/ 5.0	7.1 ± 1.08b	5.79 ± 0.37b	-	-
IAA/ADE	0 / 0	2.1 ± 0.23a*	3.4 ± 0.22a	-	-
	0.1/50.0	7.2 ± 0.96cd	4.7 ± 0.28b	-	70
IAA/AH	0.1/50.0	5.2 ± 0.48bc	4.8 ± 0.26b	-	70
IAA/Kn	0.1/ 2.0	3.1 ± 0.23ab	5.1 ± 0.34b	-	-
IAA/BAP	0.1/ 2.0	6.6 ± 0.64cd	5.3 ± 0.36b	80	80
IAA/zeatin	0.1/ 2.0	8.2 ± 0.97d	5.6 ± 0.35b	20	30

Table 2. Effects on NAA and IBA on rooting of *in vitro* produced shoots (shoots with callus) of *H. procumbens* after 4 weeks of culture on MS medium. Values represent mean ± SE (n = 20); means followed by different letters within a column are significantly different at $P < 0.05$ using Tukey-Kramer multiple comparisons test.

Phytohormones	Concentrations [mg dm^{-3}]	Root number	Root length [cm]	Rooting [%]	Callus [%]
NAA	0.0	1.2 ± 0.20a	0.2 ± 0.05a	10	60
	0.5	9.1 ± 0.75b	1.7 ± 0.19b	100	100
	1.0	14.6 ± 1.25c	2.1 ± 0.13b	100	100
	1.5	20.6 ± 1.03d	3.1 ± 0.17c	100	100
	2.0	20.6 ± 0.92d	3.0 ± 0.23c	100	100
	2.5	22.2 ± 0.85d	3.4 ± 0.13c	100	100
IBA	0.0	1.0 ± 0.24a	0.23 ± 0.04a	15	-
	0.5	11.0 ± 0.47b	0.84 ± 0.07b	70	-
	1.0	21.1 ± 0.69c	1.61 ± 0.06c	100	-
	1.5	25.4 ± 0.81d	1.43 ± 0.05c	100	-
	2.0	28.3 ± 0.65e	1.68 ± 0.08c	100	-
	2.5	30.5 ± 0.61e	2.81 ± 0.06d	100	-

undesirable as it may lead to the formation of genetically unstable plantlets. On the basis of the data it is recommended that a combination of IAA (0.1 mg dm^{-3}) and Kn (5.0 mg dm^{-3}) may be more suitable than the other cytokinins for the induction of axillary shoots without the intervention of callus growth in this species.

Shoots greater than 5 mm were transferred to MS medium with Gamborg's vitamins supplemented with IBA or NAA (*cf.* Materials and methods) for rooting. Root induction was observed in all the treatments, except the control, where only 10 to 15 % of shoots produced roots (Table 2). In general, shoots grown either with IBA or NAA produced root primordia at the base after two weeks of culture. However, the shoots treated with NAA showed 100 % rooting and developed callus at the shoot base. The tendency of NAA on callus formation during *in vitro* root development was also reported in *Eupatorium triplinerve* (Martin 2003). A similar problem of callus formation was also encountered by earlier workers (Dunstan 1981, Arena and Caso 1992). On the other hand, the treatment with IBA resulted in root induction without the callus development. The positive effect of IBA on root induction was reported in *Sesbania rostrata* (Jha *et al.* 2004), *Holarrhena antidysenterica*

(Mallikarjuna and Rajendrudu 2007) and *Ocimum basilicum* (Siddique and Anis 2007).

Acclimatization of *in vitro* developed plantlets was difficult, as some of the plantlets had hyperhydric shoots. Most of these plantlets did not survive. Nevertheless, 40 % of the plantlets survived hardening. Future research should be directed towards reducing hyperhydricity. These include exposing *in vitro* developed plantlets to progressively reduced humidity, bottom cooling of culture jars and changing of salts. Despite problems encountered, the survival rate can be improved with further refinements in the hardening procedure. The micropropagation protocol for plantlets reported herein provides a starting point for the conservation of *H. procumbens* and possibly an alternative future source for the bioactive compounds.

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