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ORIGINAL PAPER

In vitro tuberization and plant regeneration from multinodal segment culture of *Habenaria bractescens* Lindl., an Argentinean wetland orchid

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Abstract Tuberization in many terrestrial orchids represents the most important physiological process for reproduction and survival. An in vitro controlled and reproducible tuberization and plant regeneration system was designed for *Habenaria bractescens*. Multinodal segments were incubated on Murashige and Skoog (MS) medium supplemented with different concentrations of N^6-benzylaminopurine (BAP) and sucrose. After 45 days, the explants developed root tubers in vitro in 8 of the 12 media assayed. MS medium with 87.6 mM sucrose plus 4.4 μM BAP was one of the most effective for stimulating root tubers. Plants derived from in vitro root tubers were successfully transferred to the greenhouse without any acclimatization. The morphology and anatomy of the regenerated underground organs were examined to find identifying and distinguishing features. The protocol to regenerate root tubers of *H. bractescens* will be useful to study the basic aspects and control of tuberization and to carry out restoration programs.

Keywords *Habenaria bractescens* · In vitro tuberization · Orchids · Root tuber anatomy

Introduction

The Orchidaceae is probably the largest family of flowering plants (Dressler 1993). It comprises 796 genera and 17,500 species (Mabberley 1993) and is distributed in tropical, subtropical, temperate and cold regions. The Argentinean orchid flora is represented by 74 genera and 237 species. The Province of Corrientes (northeastern Argentina) contains 77 species in 37 orchid genera (Correa 1996).

Habenaria is an orchid genus of pantropical distribution with about 600 species (Hoehne 1940), mainly from perennial, terrestrial and wetland habits. In Argentina, 21 species of *Habenaria* are known, four of these, including *H. bractescens* Lindl., inhabit the Iberá macrosystem.

The Iberá macrosystem, a protected natural area, is the second-largest wetland ecosystem in South America, and supports 57% of the 2,640 plant species documented in the Province of Corrientes (Tressens and Arbo 2002). As the expansion of agricultural lands negatively impacts this protected area, orchids face particular endangerment because they are especially vulnerable plant families to alterations of their habitat (Weston et al. 2005). In particular, they have specific requirements for germination that depend on associations with specific mycorrhizal fungi (Zettler 1997). In addition, *Habenaria bractescens* is rare even in its natural habitat (Johnson 2001). In this context, we need to develop effective propagation methods for these species to ensure higher survival when the plants are reintroduced in the field for environmental restoration.

Many terrestrial and wetland orchids form storage organs, such as root tubers and rhizomes, which are important in their propagation (Pridgeon et al. 1999, 2001, 2003). Root tubers are typical vegetative plant propagules of Orchidoideae subfamily, which can survive in dry or cold conditions as dormant organs. These storage organs are very common in Orchidinae subtribe group 2 (Habenariinae sen. auct.) in the Orchideae tribe (Pridgeon et al. 2001).

Habenaria bractescens inhabits wetland environments and is characterized by the production of resupinate white

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flowers with a fringed lip and long slender spur, inserted in a terminal, pauciflorous inflorescence (Fig. 1a, b). *H. bractescens* has three types of underground organs: absorbing roots, droppers and root tubers (Fig. 1c). Absorbing roots are those that possess a typical structure consisting of a radical meristem tip with a root cap, followed by cell division and elongation zones, a root hair zone, and a maturation zone where lateral roots originate. Droppers are organs that grow downward, either vertically or obliquely, and terminate in a root tuber. Root tubers are swollen storage roots that bear shoot buds (Bell 1993; Pridgeon and Chase 1995). The formation of this storage organ is related to a phenomenon known as tuberization.

Tuberization is the physiological process whereby a stem section or a root undergoes morphological and biochemical changes to become a special storage organ (Melis

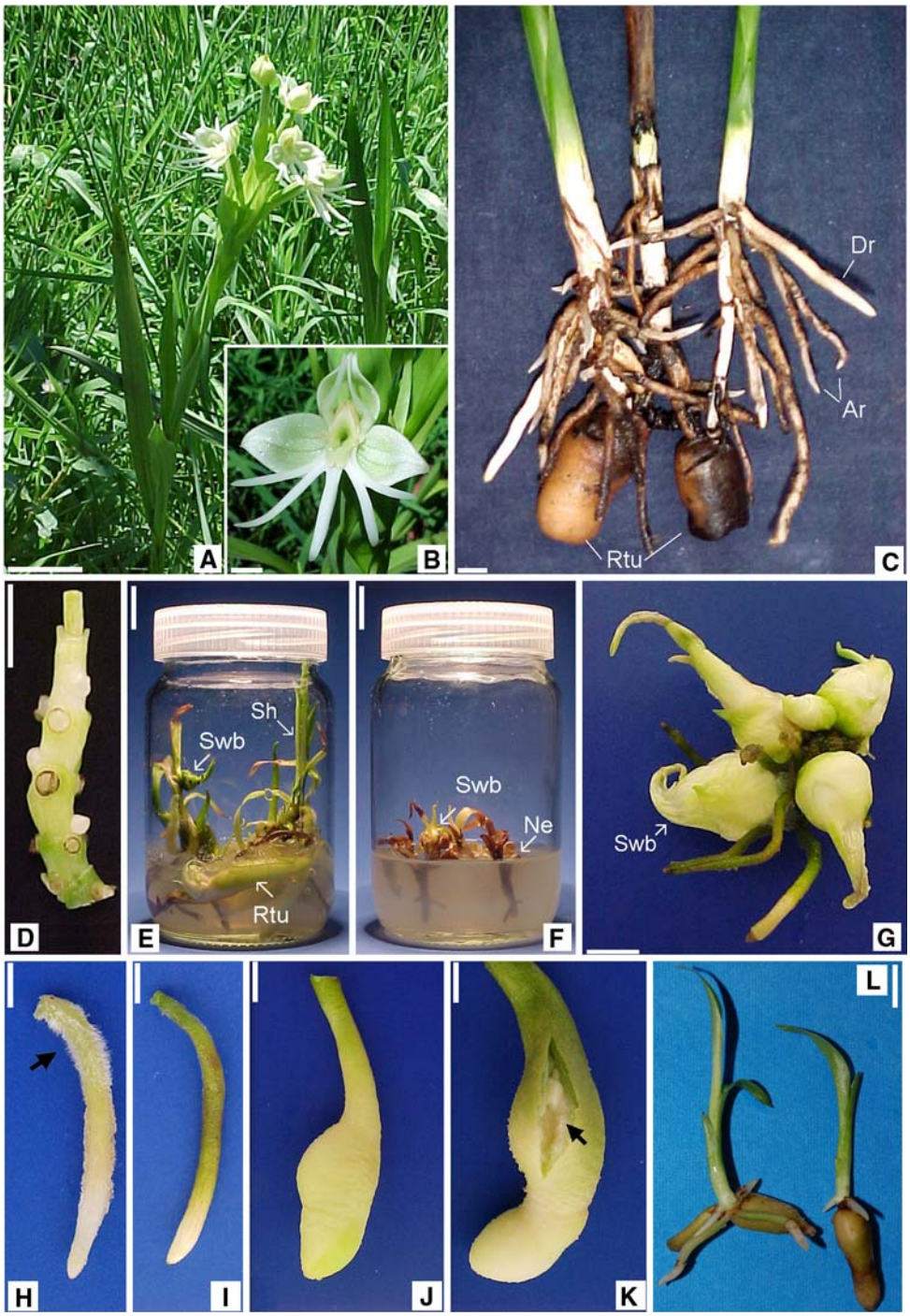

Fig. 1 Aerial and underground organs of *Habenaria bractescens* in the wild and regenerated in vivo and in vitro. **a** Plants growing in the wild in the Iberá macrosystem, bar = 10 cm; **b** flower, bar = 1 cm; **c** in vivo underground organs, bar = 1 cm; **d** in vitro multinodal segment used as the explant, bar = 0.5 cm; **e** morphogenesis after 45 d culture of multinodal segment on MS basal medium with 87.6 mM sucrose plus 4.4 μM BAP, bar = 1.5 cm; **f** morphogenesis after 45 d culture of multinodal segment on MS basal medium with 292.1 mM sucrose plus 44.4 μM BAP, bar = 1.5 cm; **g** in vitro swollen buds, bar = 1 cm; **h** absorbing root with abundant root hairs (arrow) regenerated in vitro, bar = 0.5 cm; **i** in vitro dropper, bar = 0.5 cm; **j** in vitro root tuber, bar = 0.5 cm; **k** in vitro root tuber with an emerging bud indicated by arrow, bar = 0.5 cm; **l** sprouted and rooted in vitro root tubers, bar = 1 cm. Ar: absorbing roots; Dr: dropper; Ne: necrotic explant; Sh: shoot; Swb: swollen buds; Rtu: root tubers

and van Staden 1984; Sarkar 2008). There are several reports about in vitro formation of different storage organs in monocots, e.g., in Amaryllidaceae (Ilczuk et al. 2005), Alliaceae (Goleniowski et al. 2001), Araceae (Ndoumou et al. 1995), Colchicaceae (Ghosh et al. 2007), Dioscoreaceae (Sengupta et al. 1984; Oyono et al. 2007) and Zingiberaceae (Shirgurkar et al. 2001). However, in spite of the great number of Orchidaceae that undergo this physiological phenomenon, there are very few reports of the promotion of storage organs in vitro in these species.

The induction of in vitro tuberization has been reported in some Orchidoideae subfamily species. Stewart and Kane (2006) stimulated root tuber development in *Habenaria macroceratitis* through photoperiodic control. In *H. radiata*, root tubers differentiated after extended culture on Linsmaier and Skoog basal medium (Shimada et al. 2001) or in Hyponex® medium (Yamamoto et al. 2001). The basal medium has also been modified as a strategy to induce tuberization in *Orchis papilionacea* (Pedroso and Pais 1992), *Ophrys lutea* and *O. speculum* (Barroso et al. 1990). The addition of paclobutrazol (PBZ) to the basal medium promoted root tuber formation in *Pterostylis sanguinea*, *Diuris laxiflora* and *Microtis media* seedlings (Hollick et al. 2001), as well as jasmonic acid (JA) supplementation in *P. sanguinea* protocorms (Debeljak et al. 2002). However, detailed information on the biology, physiology and biochemistry of root tubers is limited.

Induction and development of storage organs involve many extrinsic (photoperiod, temperature, nitrogen level, and exogenous sucrose or plant growth regulators [PGRs] application) and intrinsic factors (genotype, physiological age, hormonal contents and their balance) (Jackson 1999; Fernie and Willmitzer 2001). Many reports in the literature describe the importance of PGRs on tuberization. Cytokinins and gibberellins (GAs) are the most important phytohormones involved in the regulation of storage organ formation (Sarkar 2008). GAs are well known to have an inhibitory effect on storage organ formation (Vreugdenhil and Sergeeva 1999). On the contrary, cytokinins have been considered to be involved in the development of the storage organ by promoting cell division in the growing tuber (Fernie and Willmitzer 2001).

Based on research on potato (*Solanum tuberosum*), the most widely studied tuber species, important progress in understanding the tuberization process has been made (Melis and van Staden 1984; Struik et al. 1999; Sarkar 2008).

The aim of this study was to develop a reliable method to form root tubers and regenerate plants of *H. bractescens* using in vitro tissue culture. For this purpose, we evaluated the effect of different concentrations and combinations of cytokinin (BAP) and sucrose. We also compared the anatomy and morphology of in vitro absorbing roots, droppers and root tubers with in vivo homologous organs. In addition, we cut serial sections through droppers to obtain histological details of their vascularization and to establish the main differences between droppers and absorbing roots and the relationship with the formation of the root tubers.

Materials and methods

Seed source and plant material

Plants of *H. bractescens* Lindl. (Fig. 1a) were collected in March 2004 in the Iberá macrosystem (region placed in the Province of Corrientes, Argentina, ranging from northeast to southwest, from approximately 56°30′ E to 59°00′ W to 27°50′ N to 29°00′ S). Voucher specimens were deposited at the herbarium of the Instituto de Botánica del Nordeste (CTES). Plants were grown in 10 l plastic pots containing a mixture of *Sphagnum* moss, humus and perlite (5:3:2) and kept flooded during their growth. The plants flowered from November to January. Capsules with fertile seeds were obtained after artificial pollination of flowers using pollinea of flowers from the same inflorescence. Capsules were harvested in February 2005 prior to dehiscence and were surface sterilized for 1 min in 70% ethanol solution and then immersed in a solution of NaOCl (1.7% available Cl) with the addition of 0.03% Triton-100X® for 30 min, followed by several rinses in sterile deionized water. Capsules were opened on sterile Petri dishes in a laminar flow cabinet, and seeds were spread in 355 ml glass flasks (1,012 ± 40 seeds per glass flasks) containing 80 ml of half-strength Murashige and Skoog basal medium (MS) (Murashige and Skoog 1962) with 87.6 mM sucrose. The pH of the medium was adjusted to 5.5 using either HCl or KOH before the addition of 0.65% Sigma agar (A-1296) and autoclaving at 121°C for 20 min. Cultures were covered with Resinite AF-50® film (Casco S. A. C. Company, Buenos Aires) and incubated in a growth room at 27 ± 2°C with a 14 h photoperiod and an irradiance of 116 μmol m^{-2} s^{-1} provided by cool white fluorescent lamps. After 4 months, plants were transferred to full-strength MS basal medium. Multinodal stem segments (at least five nodes and ~2 cm long, Fig. 1d), dissected from *H. bractescens* plants multiplied in vitro, were used as a source of explants. Possible topophysis effects were avoided by using only the basal portion of the stem.

Culture conditions for root tuber formation

Multinodal segments were cultured in 355 ml glass flasks containing 100 ml of full-strength MS basal medium with different sucrose concentrations (87.6, 146.7 or 292.1 mM) alone or combined with 0, 4.4, 22.2 or 44.4 μM BAP.

Cultures were covered with Resinite AF-50® film (Casco S. A. C. Co.) and incubated as before.

Plant regeneration

After 45 d culture, all multinodal segments grown on different concentrations and combinations of BAP and sucrose had regenerated shoots (upright leafy shoots), swollen buds (similar to pseudobulbs), and root tubers, all of which were then transferred separately to MS basal medium supplemented with 87.6 mM sucrose to complete plant regeneration. After 60 d subculture, plants derived from in vitro shoots, swollen buds and root tubers were removed from glass flasks, soaked in tap water to remove the remaining culture medium and rinsed carefully. They were submerged in fungicide solution for 30 min, then transplanted into a mixture of *Sphagnum* moss, humus and perlite (5:3:2) in plastic containers (15 cm diameter). Plants derived from in vitro shoots and swollen buds were incubated in a humidity chamber at 90% relative humidity (RH) for 7 days and an irradiance of 160 μmol m^{-2} s^{-1}. The RH was then gradually decreased to 70%. After 30 days, the acclimatizated plants were transferred to the greenhouse under 80% sunlight (aluminium shading net). Plants derived from in vitro root tubers were transferred to greenhouse conditions without previous acclimatization.

Morphological and anatomical analysis

In order to identify and differentiate absorbing roots, droppers and root tubers, morphological and anatomical analyses were performed. Samples obtained from in vivo and in vitro plants were histologically examined.

Eight months post- planting, plants grown under greenhouse conditions were carefully uprooted and gently cleaned with tap water. In vivo samples were hand-sectioned with a razor blade, sections and then stained with safranin (C.I. 50240)—astra blue (Luque et al. 1996), and observed under a light microscope (Olympus CH 30, Tokyo, Japan).

In vitro plant material was sampled after 45 d of culture. All samples were fixed in FAA (formalin, acetic acid, 70% ethanol, 5:5:90) and dehydrated with Deshidratante histológico BIOPUR® S.R.L. (Gonzalez and Cristóbal 1997), embedded in paraffin (Johansen 1940), and serially sectioned (12 μm thick) with a rotatory microtome (Microm, Walldorf, Germany). Sections were stained as mentioned and mounted in synthetic Canada balsam.

Illustrations were drawn using a camera lucida on a light microscope (Olympus BX 50, Tokyo, Japan), and photographs were taken using an optical microscope (Leica DM Wetzlar, Germany) equipped with a photographic camera (Canon Power Shot S50 AIAF, Tokyo Japan).

Experimental design

The experiment was arranged in a completely randomized design (explant × 12 different media). Each treatment consisted of 15 replicates (culture vessels) with three explants per glass flask, making 45 explants per treatment.

Data recording and statistical analysis

Growth response data, recorded after 45 d culture, included percentage of necrotic explants (those explants that turned brown and finally died), number and percentage of explants with root tubers, relative proportion of underground organs (absorbing roots, droppers, root tubers or root tubers with bud) regenerated per explant, percentage of explants that formed shoots or swollen buds, and number of shoots or swollen buds per explant. Structures thicker than 8 mm in their distal portion were scored as root tubers. Smaller structures were recorded as droppers. After 60 d culture, the percentage of shoots, swollen buds or root tubers that produced plants was calculated (plant regeneration frequency). Sixty days after transplanting, the ex vitro surviving plantlets derived from shoots, swollen buds or root tubers were counted.

Data were subjected to one-way analysis of variance (ANOVA), and the means were compared by Duncan's multiple comparison test ($P \leq 0.05$; $P \leq 0.01$) using InfoStat software professional version 1.1 (InfoStat 2002). Means are presented with standard error (±SE).

Results

In vivo and in vitro regeneration of underground organs

Habenaria bractescens plants that were grown in plastic containers developed underground organs mainly from the basal region of the stem. Eight months after planting, these plants produced absorbing roots, droppers and droppers with root tubers. Many in vivo root tubers were attached to the stem via a thin, peduncle-like dropper.

After 15 d culture, multinodal segments sprouted in vitro and directly produced absorbing roots and droppers. Typical underground organs, similar to those produced under in vivo conditions, were noted after 45 d (Fig. 1e, h–k).

The highest percentages of necrotic explants (52–80%) were obtained in media with the highest sucrose concentration (292.1 mM). However, supplemental BAP significantly increased the percentage of necrotic explants only at the highest sucrose concentration media (Table 1). The increase in sucrose concentration induced a high occurrence of necrotic explants, explants with small shoots

Table 1 Effect of N^6-benzylaminopurine (BAP) and sucrose concentration alone and in combination on percentage of explants of *Habenaria bractescens* displaying necrosis after 45 d of culture

BAP (μM)	% Necrotic explants[a]		
	Sucrose (mM)		
	87.6	146.7	292.1
0	0 A	0 A	51.8 ± 5.7 D
4.4	0 A	20.6 ± 10.4 C	67.4 ± 1.3 E
22.2	2.8 ± 2.8[b] AB	17.0 ± 2.9 BC	79.8 ± 3.6 E
44.4	14.9 ± 3.3 ABC	20.5 ± 8.3 C	73.63 ± 6.1 E

[a] Explants that turned brown and died

[b] Numerals indicate mean values ± SE

Different letters within columns and rows indicate significant differences according to Duncan's multiple comparison test ($P \leq 0.05$)

and swollen buds, reduced leaf area, and fewer underground organs (Fig. 1f).

Typical *H. bractescens* underground organs were regenerated in vitro: absorbing roots with abundant root hairs (Fig. 1h), thin root-like structures called droppers (Fig. 1i), and root tubers in different maturation stages. It was possible to observe immature root tubers derived from the swollen apical portion of the dropper (Fig. 1j) and mature root tubers with an emerging bud (Fig. 1k). These root tubers originated from a slender dropper or from a very short dropper that emerged from the stem. Two weeks after root tuber formation, root tubers sprouted from the expanding bud (Fig. 1l). The relative proportion of underground organs regenerated per explant varied depending on culture media (Fig. 2). In all cases, 2–9 absorbing roots were observed on each explant, and in some media, absorbing roots were the only type of underground organ formed. The addition of BAP increased the number of droppers that differentiated per explant and the number of droppers that developed root tubers, except at the highest sucrose concentrations with 22.2 and 44.4 μM BAP (Fig. 2). Droppers were regenerated in 83.3% of the culture media assayed. In BAP-free medium containing 146.7 or 292.1 mM sucrose, the number of droppers per explant was reduced to 50% of that in media with 87.6 mM sucrose. In the culture media with 87.6 mM sucrose combined with the different BAP concentrations, the number of droppers per explant remained constant (two per explant). However, the presence of BAP in combination with 146.7 mM sucrose increased the number of droppers per explant to three per explant), regardless of the BAP concentration. Media with 292.1 mM sucrose and 4.4 μM BAP produced the highest number of droppers per explant; however, higher BAP concentrations inhibited dropper regeneration.

Anatomy and histology of in vivo and in vitro underground organs

The underground organ regenerated in vitro resembles the typical morphology and anatomical features of those produced in vivo. In organ types regenerated in vitro, no anatomical differences, except for length, could be attributed to the culture media. All the underground organs were shorter in media at higher concentrations of BAP and sucrose.

Absorbing root

Three areas of the root tip were recognized (Fig. 3a): the root cap, an active cell division zone, and a cell elongation zone. Next to the cell elongation zone was the absorbent root hair zone. In transverse cuts in this region, the

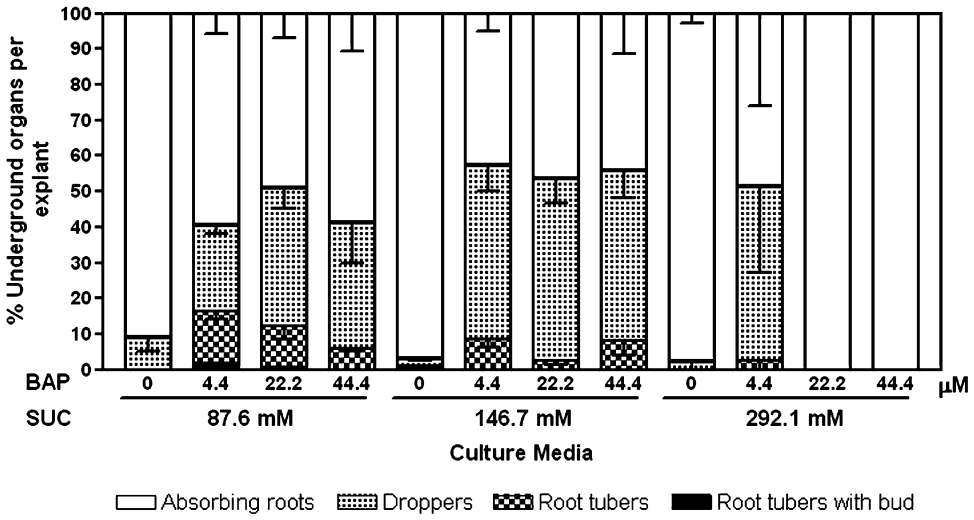

Fig. 2 Relative proportion of in vitro underground organs (absorbing roots, droppers, root tubers and root tubers with bud) regenerated per explant of *Habenaria bractescens* subjected to different concentrations and combinations of N^6-benzylaminopurine (BAP) and sucrose (SUC), after 45 d culture

epidermis developed numerous unicellular hairs (Fig. 3b). The cortex had an outer parenchymatous zone surrounding aerenchyma cells (Fig. 3b). An endodermis with Casparian strips formed the innermost layer of the cortex. The roots were polyarchs with 10–15 poles (Fig. 3b–c).

Dropper

At initial developmental stages, young droppers (Fig. 3d) were difficult to distinguish externally from absorbing roots (Fig. 3a). However, through detailed observations, the absorbing roots were seen to have a more acute radical apex (Fig. 1h) than the droppers had (Fig. 1i, j). These differences were more evident after the droppers had completed development.

The dropper epidermis was composed of a uniseriate layer of suberized cells with unicellular hairs. The cortex consisted of 2–3 layers of parenchymatous cells without intercellular spaces. Middle layers were composed of aerenchyma (Fig. 3e). The vascular cylinder was comprised of meristeles (Fig. 3k–n); and each was surrounded by a unilayered endodermis with Casparian strips (Fig. 3f). In vivo droppers had 8–10 meristeles, while those from the in vitro plants had only 2–3. Both materials had an eccentric

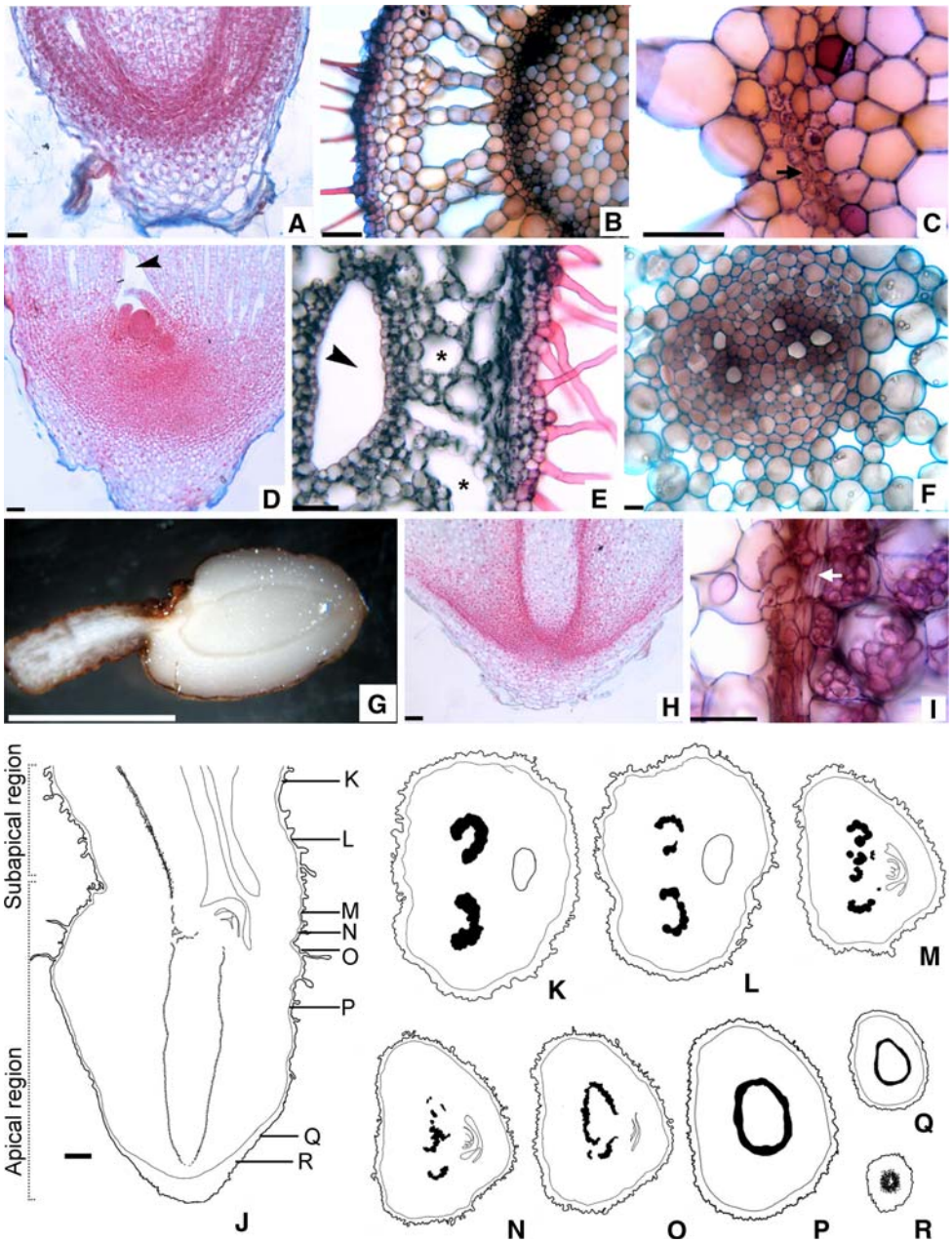

Fig. 3 Anatomy and histology of in vivo and in vitro underground organs of *Habenaria bractescens*. **a** Longitudinal section of an absorbing root; **b**, **c** transverse sections of absorbing roots, black arrow: Casparian strips; **d** longitudinal section of a dropper, arrowhead: channel; **e** air chambers (asterisks) and channel (arrowhead) in dropper aerenchyma; **f** meristele of dropper; **g** macroscopic section of a root tuber; **h** radical meristem tip of a root tuber; **i** details of cortical parenchyma and vascular cylinder with starch granules of root tuber, white arrow: Casparian strip; **j** diagram of vascularization of an in vitro dropper showing apical and subapical regions; **k**–**r** diagram of transverse sections of in vitro dropper at positions labelled in Fig. 3j. Bars = 0.1 mm, except for (**g**) where bar = 1 cm and **j**–**r** where bars = 0.5 cm

channel spanning the entire dropper from the bud to the stem (Fig. 3d–e, arrowheads; Fig. 3j–n).

Root tuber

The root tuber originated from the distal end of a dropper (Fig. 3g) between the bud and the root apical meristem (Fig. 3j, o–r). Tuberization took place when the apical portion of the dropper underwent radial expansion, maintaining the typical radical structure (Fig. 3h), and the cortical parenchyma had starch granules (Fig. 3i).

The epidermis was a single layer and had unicellular trichomes. The cortex consisted of compact parenchyma filled with simple, ovoid starch granules and had an endodermis with Casparian strips (Fig. 3i, arrow). In older in vivo tubers, the outer layers of cortex were periclinally compressed. The vascular cylinder in the in vitro root tuber had 10 alternating groups of xylem and phloem; however, the in vivo material had more xylematic poles.

In vitro tuberization

Root tuber formation was influenced by media composition (Fig. 4). At 45 d, root tuber differentiation was promoted in 8 of the 12 culture media assayed, earlier than for container-grown specimens from the Iberá macrosystem (ca. 3–4 months after planting). One of the most effective media for root tuber regeneration was the MS with 87.6 mM sucrose plus 4.4 µM BAP. Each explant produced one root tuber. Donor plants maintained on MS basal medium with 87.6 mM sucrose during 5 months also regenerated root tubers (data not shown).

Shoot and bud differentiation and plant regeneration

Axillary buds on the multinodal segments developed upright leafy shoots (Fig. 1e) and/or swollen buds (Fig. 1e–g) depending on the culture media. Shoot differentiation decreased with increasing BAP concentration regardless of the sucrose concentration (Table 2). BAP-free media did not

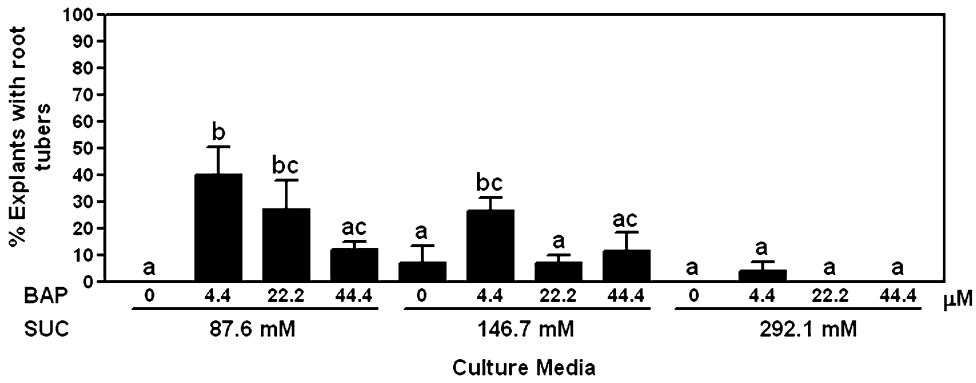

Fig. 4 Effect of different concentrations of N^6-benzylaminopurine (BAP) and sucrose (SUC) on in vitro tuberization of *Habenaria bractescens* from multinodal segments after 45 d culture

Table 2 Effect of N^6-benzylaminopurine (BAP) and sucrose alone and in combination on in vitro regeneration of upright leafy shoots (shoots) and swollen buds (pseudobulb similar structure) from multinodal segment explants of *Habenaria bractescens* cultured for 45 d

BAP (µM)	Sucrose (mM)	% Explants forming shoots	No. shoots/explant	% Explants forming swollen buds	No. swollen buds/explant
0	87.6	100.0 A	4.2 ± 1.2 A	0 C	0 D
4.4		57.1 ± 6.6[a] B	1.9 ± 0.1 BC	56.3 ± 17.5 B	3.1 ± 0.6 A
22.2		17.4 ± 4.6 C	1.0 ± 0.0 CD	88.6 ± 7.3 A	4.3 ± 0.2 A
44.4		11.3 ± 7.3 C	0.7 ± 0.3 CD	79.3 ± 3.3 AB	4.1 ± 0.3 A
0	146.7	100.0 A	2.9 ± 0.3 B	0 C	0 D
4.4		44.5 ± 7.8 B	1.0 ± 0.0 CD	73.8 ± 4.9 AB	1.9 ± 0.3 BC
22.2		20.7 ± 5.8 C	1.0 ± 0.0 CD	82.9 ± 2.9 A	4.4 ± 1.5 A
44.4		5.6 ± 5.6 C	0.3 ± 0.3 D	76.8 ± 8.2 AB	3.5 ± 0.3 AB
0	292.1	48.2 ± 5.7 B	1.4 ± 0.2 CD	0 C	0 D
4.4		10.7 ± 6.4 C	0.7 ± 0.3 CD	20.0 ± 10.0 C	1.1 ± 0.6 CD
22.2		12.1 ± 8.0 C	0.7 ± 0.3 CD	12.1 ± 8.0 C	0.7 ± 0.3 CD
44.4		2.6 ± 2.6 C	0.0 ± 0.3 D	23.8 ± 8.5 C	1.9 ± 0.6 BC

[a] Numerals indicate mean values ± SE

Different letters within columns indicate significant differences according to Duncan's multiple comparison test ($P \leq 0.05$)

promote the growth of swollen buds, regardless of sucrose concentration (Table 2). BAP supplementation stimulated the differentiation of swollen buds; however, in media with 292.2 mM sucrose, this capacity decreased (Table 2). The highest sucrose concentration also had a similar depressive effect on the number of shoots and the number of swollen buds regenerated per explant (Table 2).

After 60 d culture, upright leafy shoots produced on BAP-free media or media supplemented with 4.4 or 22.2 μM BAP and sucrose at the three concentrations evaluated were able to develop complete plants when transferred to MS basal medium; however, none of the shoots obtained with 44.4 μM BAP regenerated plants regardless of the sucrose concentration (Table 3).

Swollen buds that differentiated in the media with different concentrations and combinations of BAP and sucrose were also able to regenerate plants. The percentage regeneration from swollen buds produced on MS basal medium plus 146.7 mM sucrose and 4.4 μM BAP statistically differed from swollen buds produced on the same medium with the same sucrose level but with 44.4 μM BAP and with MS basal media containing 292 mM sucrose regardless of the BAP concentration (Table 3). In all cases, root tubers sprouted and rooted (Fig. 1l) and regenerated vigorous and healthy plants (Table 3).

Plants cultured in plastic containers derived from either shoots, swollen buds or root tubers were similar to the plants grown under wild conditions; however, the ex vitro survival ratio depended significantly on the explant origin (Table 4).

Table 3 Mean percentages of in vitro plant regeneration of *Habenaria bractescens* from root tubers, upright leafy shoots (shoots) and swollen buds (pseudobulb similar structure) differentiated previously on different concentrations and combinations of N^6-benzylaminopurine (BAP) and sucrose and transferred individually to MS basal medium, after 60 d subculture

BAP (μM)	Sucrose (mM)	% Root tubers producing plants	% Shoots producing plants	% Swollen buds producing plants
0	87.6	–	100.0 A	–
4.4		100.0	98.2 ± 1.8[a] A	71.3 ± 9.0 AB
22.2		100.0	100.0 A	78.3 ± 6.0 AB
44.4		100.0	0 B	79.2 ± 4.2 AB
0	146.7	100.0	100.0 A	–
4.4		100.0	100.0 A	100.0 A
22.2		100.0	100.0 A	85.6 ± 7.4 AB
44.4		100.0	0 B	65.0 ± 6.6 B
0	292.1	–	95.8 ± 4.2 A	–
4.4		100	100 A	58.3 ± 25.0 B
22.2		–	100 A	60.0 ± 24.5 B
44.4		–	0 B	62.5 ± 23.9 B

[a] Numerals indicate mean values ± SE

Different letters within columns indicate significant differences according to Duncan's multiple comparison test ($P \leq 0.05$)

Table 4 Mean percentages of ex vitro plantlet survival of *Habenaria bractescens* from root tubers, upright leafy shoots (shoots) and swollen buds (pseudobulb similar structure) differentiated previously on different concentrations and combinations of N^6-benzylaminopurine (BAP) and sucrose, after 60 d of transplanting

BAP (μM)	Sucrose (mM)	Ex vitro survival of plantlets (%)		
		Derived from root tubers[a]	Derived from shoots[a]	Derived from swollen buds[a]
0	87.6	–	84.4 ± 4.4 A	–
4.4		97.9 ± 2.1[b] A	82.2 ± 2.2 A	60.0 ± 5.8 A
22.2		95.5 ± 2.2 A	80.0 ± 6.7 A	55.6 ± 1.4 A
44.4		93.3 ± 3.8 A	–	53.7 ± 4.9 A
0	146.7	97.6 ± 2.4 A	80.0 ± 3.9 A	–
4.4		95.3 ± 2.4 A	80.0 ± 3.9 A	56.7 ± 3.3 A
22.2		95.3 ± 2.4 A	77.8 ± 4.5 A	54.2 ± 2.4 A
44.4		92.9 ± 4.1 A	–	51.9 ± 3.7 A
0	292.1	–	77.8 ± 4.5 A	–
4.4		94.2 ± 2.9 A	75.5 ± 2.2 A	53.3 ± 3.3 A
22.2		–	73.3 ± 3.8 A	52.8 ± 2.8 A
44.4		–	–	48.1 ± 3.7 A
Overall mean[c]		95.3 ± 0.6 A	79.0 ± 1.1 B	54.0 ± 1.1 C

[a] Different letters within columns indicate significant differences according to Duncan's multiple comparison test ($P \leq 0.05$)

[b] Numerals indicate mean values ± SE

[c] Different letters within row indicate significant differences according to Duncan's multiple comparison test ($P \leq 0.01$)

Discussion

Terrestrial orchids can be propagated through several in vitro procedures (symbiotic or asymbiotic germination, nodal segment multiplication, and somatic embryogenesis) to produce a large number of healthy plants. Unfortunately, these plants are fragile and survive poorly after transplanting in comparison to plants derived by the sprouting of robust or field-hardy propagules such as rhizomes or root tubers. Propagation techniques can exploit this attribute to ensure the successful survival of the plants transplanted to soil. Thus, the production of storage organs would be the ideal method for restoration or reintroduction programs of these orchid species. Importantly, in vitro culture procedures are compatible with sophisticated techniques currently used to increase genetic diversity. Two successful efforts for restoration and habitat recovery of *Habenaria radiata* in Japan involved root tuber production after 3–4 months culture on basal medium (Shimada et al. 2001;

Yamamoto et al. 2001). We have demonstrated in the present study that early tuberization of multinodal segments of *H. bractescens* can be stimulated reliably in the presence of cytokinin and sucrose. The subsequently regenerated plants can then be used for restoration.

Root tubers also formed on *H. bractescens* plants cultured on MS basal medium for 5 months, and no subculturing on fresh media was needed. We believe that, in this case, tuberization was induced by either water or nutrient-deficiency stress caused by depletion of the culture medium. However, induction of root tuber formation using this method is not recommended; it takes too long, is difficult to control, and lacks repeatability.

The role of cytokinins on tuberization and the promotive effect of sucrose on storage organ formation have been previously reported (Jackson 1999; Fernie and Willmitzer 2001; Sarkar 2008). BAP has been one of the most successfully used cytokinins for tuberization induction in several species (Zhou et al. 1999; Piao et al. 2003; Omokolo et al. 2003; Poornima and Ravishankar Rai 2007). In orchids, BAP has frequently promoted in vitro rhizome regeneration. For example, rhizome differentiation was enhanced by BAP alone or by BAP with α-naphthalene acetic acid (NAA) in *Geodorum densiflorum* (Roy and Banerjee 2002). Similarly, BAP enhanced rhizome proliferation enabling efficient mass propagation of *Cymbidium ensifolium* (Chang and Chang 1998).

Induction of storage organ formation in the presence of other plant growth regulators has been reported. Paclobutrazol (PBZ), evaluated for three Orchidoideae species, stimulates early tuberization (after 8 weeks of culture) in only two species, *Diuris laxiflora* and *Microtis media* (Hollick et al. 2001). Storage organ formation was also enhanced in *Pterostylis sanguinea* following jasmonic acid (JA) application. However, JA did not cause a similar increase in dropper number, root tuber diameter, or root tuber fresh mass. In the present study, BAP addition to the culture medium promoted root tuber formation in *H. bractescens* although the efficiency varied with both BAP and sucrose concentrations in the culture medium. The inductive effect of BAP is more remarkable if droppers are considered to be potential root tubers. Probably if the explants had been cultured for more than 45 days, the droppers would have developed into root tubers, or we would have needed to subculture them using fresh induction media to stimulate their tuberization.

In vitro tuberization is also highly dependent on sucrose concentration (Xu et al. 1998); high sugar concentrations are generally necessary (Hussey and Stacey 1984; Jackson 1999; Fernie and Willmitzer 2001). On the other hand, tuberization was enhanced at lower sugar levels than the typical inductive concentrations of sucrose (175.3–233.7 mM) for three terrestrial Orchidoideae species, *Pterostylis sanguinea* (29.2 mM sucrose), *Habenaria radiata* (43.8 mM sucrose) and *H. bractescens* (87.6 mM sucrose).

The morphology and anatomy of underground organs that differentiated under in vivo conditions have been reported for diverse orchids (Kumazawa 1958; Bell 1993; Stern 1997; Loizaga de Castro and Arbo 2005). Our paper, however, is the first report on the morphology, anatomy and histology of in vitro regenerated storage organs as compared with in vivo homologous organs in orchid species. In these previous reports, authors used different names for the same structure. For example, Kumazawa (1958) referred to the dropper as a stoloniferous sinker. Our analysis of dropper anatomy and vascularization showed the radical nature of its distal pole, from which the root tuber of *H. bractescens* originates, as mentioned for other Habenariinae sen. auct. species such as *Herminium monorchis* (Bell 1993). Stern (1997) recognized and described two roots types: absorbing roots and root tubers, without mentioning the droppers. His description of aberrant absorbing roots for *Habenaria arenaria* is consistent with our observations of droppers. Stern (1997) also described two root tuber types. According to the present work, *H. bractescens* root tubers would be included in the group bearing a single vascular cylinder. Our general observations are consistent with the previous anatomical descriptions of in vivo materials of *H. bractescens* (Loizaga de Castro and Arbo 2005). In addition, our results confirm that in vitro tuberization occurs in the same way as in vivo and that the regenerated organs are anatomically and morphologically identical.

The differentiation of upright leafy shoots was promoted when BAP was used at low concentrations or was absent. Plant regeneration from shoot culture was inhibited on media with the highest BAP concentration. Similarly, high BAP concentrations inhibited root and shoot regeneration in other species (Roy and Banerjee 2002; Medina et al. 2007). In the present study, however, swollen buds were only regenerated when BAP was present. Although swollen buds were able to regenerate plants on MS basal medium regardless of the previous induction medium, the frequency was variable. *Habenaria bractescens* naturally produces only upright leafy shoots, but when cultured in vitro on BAP-supplemented media, the plant was able to differentiate structures similar to pseudobulbs. Similar sucrose concentrations have been used to induce pseudobulbs in *Oncidum varicosum* (Rego-Oliveira et al. 2003). In *Dactylorhiza* species, BAP has also been combined with NAA to induce pseudobulb differentiation (Wotavová-Novotná et al. 2007).

Plant regeneration from root tuber subculture on MS basal medium was optimal (100%), regardless of previous induction culture media. In general, although the frequency of plant regeneration was fairly high (58.3–100%), it

depended on the explant origin and the previous induction medium. It is important to note that normal shoots and root tubers with buds readily sprouted and rooted on MS basal medium. The low regeneration of plants from swollen bud culture was due to the fact that only 14.4–41.7% of the swollen buds sprouted or rooted; most finally died as a consequence of oxidative browning. This situation might be related to the bud maturation stage, dormancy or lack of appropriate conditions for growth and development. As reported for other species, the plant regeneration potential and ex vitro plantlets survival varies with the explant source (Agarwal and Ranu 2000; Franklin and Dias 2006) or age (Prakash and Gurumurthi 2004).

In summary, the protocol for in vitro root tuber formation for *H. bractescens* described here provides a novel system for the controlled and reliable development of root tubers. In vitro root tuber formation was promoted by eight culture media tested although the efficiency differed significantly depending on the medium. Plants could be regenerated at high frequency, but the rate was considerably influenced by the explant source and the previous inductive culture media. Our anatomical and histological studies identified useful features to distinguish the different in vivo and in vitro underground organs and confirmed normal differentiation under in vitro conditions.

This protocol could be used for basic developmental studies, propagation or for environmental restoration programs.

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