In vitro propagation via organogenesis and synthetic seeds of *Urginea altissima* (L.f.) Baker: a threatened medicinal plant

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Received: 15 June 2017 / Accepted: 4 December 2017 / Published online: 12 December 2017
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Abstract

Efficient in vitro propagation systems via organogenesis and synthetic seeds were developed for the first time for conservation and commercial propagation from leaf or longitudinal thin cell layer (ITCL) leaf or shoot-tip explants of *Urginea altissima*. Various plant growth regulators and phloroglucinol were used in semi-solid and liquid Murashige and Skoog (MS) medium to establish multiplication of shoots and roots for in vitro regeneration. Of the various treatments, the highest number of shoots (17.4 per ITCL leaf explant) was obtained on liquid MS medium supplemented with 10 μM *meta*-Topolin (*mT*) and 2 μM benzyladenine followed by transferal to semi-solid MS media. The shoot tips were encapsulated with liquid MS medium plus 3% (w/v) sodium alginate and 100 mM calcium chloride. Adventitious shoot regeneration (91.0%; 12.6 shoots per synthetic seed) of synthetic seeds was achieved on semi-solid MS medium supplemented with 10 μM *mT* and 2 μM naphthaleneacetic acid (NAA) after 15 days of storage in darkness at 25 ± 2 °C. Regenerated shoots rooted (9.8 roots per shoot; 6.5 cm long) efficiently when transferred to 5 μM indole-3-butyric acid and 2.5 μM NAA. All the plantlets were successfully acclimatized (100%) in a vermiculite:soil (1:1 v/v) mixture in the greenhouse.

Keywords Ex situ conservation · In vitro regeneration · Liquid culture · Medicinal plant · Synthetic seeds

Introduction

Many of the Hyacinthaceae genera, including *Urginea*, have medicinal and ornamental potential (McCartan and Van Staden 1999; SANBI 2017). *Urginea altissima* (L.f.) Baker (Hyacinthaceae) is a threatened perennial bulbous medicinal plant (Williams et al. 2016). It is popularly known as squills. The plant is used traditionally for the treatment of influenza, asthma, bronchitis, warts, rheumatism, cancer, congestive heart failure and cardiac arrhythmia (Watt and Breyer-Brandwijk 1962; Hutchings 1989; Hutchings et al. 1996; Langat et al. 2013). It is also used as an abortifacient and emmenagogue (Hutchings 1989; Langat et al. 2013). The plant produces various bioactive compounds, viz. bufadienolides, scilliglaucoside, 1 β , 6 α -dihydroxy-4(15)-eudesmene and a polyhydroxylated furan derivative, polybotrin (Langat

et al. 2013). The ornamental and medicinal important wild populations of *Urginea altissima* are over-exploited and the plant has thus been categorized as a threatened species in the Red Data List of South African Plants (Williams et al. 2016; SANBI 2017). Although micropropagation protocols have been developed for other *Urginea* species (Jha et al. 1984; Jha and Sen 1986; El Grari and Backhaus 1987; Stojakowska 1993), in vitro propagation for *U. altissima* is yet to be investigated.

In vitro regeneration techniques offer an efficient method for propagation of threatened medicinal plants (Jha et al. 1984; El Grari and Backhaus 1987; Stojakowska 1993; McCartan and Van Staden 1999; Shahzad and Shaheen 2013; Yanthan et al. 2017). In vitro regeneration via direct and indirect organogenesis through semi-solid and liquid culture and synthetic seeds have been identified as a potential resource for ex situ conservation and commercial applications, continuous production of plants without any seasonal limitations, in vitro gene banking and exchange of germplasm across international borders. The technique was also useful for new drug development, clinical research in the fields of pharmacology and medicine as well as production of bioactive compounds (Sajc et al. 2000; Nyende

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et al. 2003; Manjkhola et al. 2005; Georgiev et al. 2011; Kumari et al. 2016). Therefore, in vitro regeneration via organogenesis (direct and indirect) from semi-solid and liquid culture and synthetic seed technology could play an important role in mass propagation, germplasm conservation, ornamental industry, genetic improvement, bioactivity and pharmaceutical compound production in *U. altissima*. In the present investigation, we aim to establish simple and effective high-frequency in vitro plant regeneration protocol for *U. altissima*, using different cultural strategies including explant, culture medium, plant growth regulators and phloroglucinol for large-scale production to help in the conservation and commercial propagation of this species as an alternative to naturally grown plants.

Materials and methods

Plant material and shoot regeneration from semi-solid cultures

Young leaves of *Urginea altissima* (L.f.) Baker were collected from the Botanical Garden, University of KwaZulu-Natal, Pietermaritzburg, South Africa. Explants were decontaminated using 0.2% (w/v) aqueous HgCl_2 for 10 min followed by five rinses with sterile distilled water. Disinfected leaf explants (approximately, 15×10 mm) were used for direct and indirect shoot regeneration. The explants were cultured in solidified (8 g L^{-1} agar) MS (Murashige and Skoog 1962) medium (semi-solid MS) containing 30 g L^{-1} sucrose and supplemented with $10 \mu\text{M}$ benzyladenine (BA), *meta*-topolin (*mT*) and thidiazuron (TDZ) alone or in combination with $2 \mu\text{M}$ indole-3-acetic acid (IAA) or indole-3-butyric acid (IBA) or naphthaleneacetic acid (NAA) or phloroglucinol (PG) for direct shoot regeneration. White compact callus obtained with semi-solid MS medium containing $10 \mu\text{M}$ 2,4-D and $2 \mu\text{M}$ TDZ was established onto semi-solid MS medium containing $10 \mu\text{M}$ BA or *mT* or TDZ and in combinations with $2 \mu\text{M}$ picloram (Pic) or 2,4-dichlorophenoxyacetic acid (2,4-D) or NAA or IAA or BA or *mT* or TDZ for indirect shoot regeneration.

Shoot regeneration from liquid cultures and synthetic seeds

For direct shoot regeneration of liquid culture, longitudinal thin cell layers (ITCL) of leaf (10×2 mm from base of leaf) explants were inoculated onto liquid MS medium (20 mL in 100-mL Erlenmeyer flask) containing 30 g L^{-1} sucrose and fortified with $10 \mu\text{M}$ BA or *mT* or TDZ and combinations of $10 \mu\text{M}$ *mT* plus $2 \mu\text{M}$ BA or TDZ. The liquid cultures were maintained on an orbital shaker at 80 rpm. Liquid medium with respective PGRs was refreshed every 2 weeks once for

4 weeks. Shoot-sprouting explants from respective culture were transferred to semi-solid MS medium for 8 weeks for further development of shoot regeneration.

The synthetic seeds were prepared with MS medium plus 3% (w/v) sodium alginate (SA) and 100 mM calcium chloride ($\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$: 100 mM for 10 min exposure) from shoot tips (5–10 mm long) of optimal direct shoot regeneration liquid media containing combinations of $10 \mu\text{M}$ *mT* and $2 \mu\text{M}$ BA (Table 2). The synthetic seeds were stored in dark at $25 \pm 2^\circ\text{C}$ in a growth room or 4°C in a refrigerator (Kelvinator, Charlotte, USA) for 15 days to determine optimal temperature and viability of regeneration for germplasm conservation. Synthetic seeds were then incubated in semi-solid MS medium containing 30 g L^{-1} sucrose and supplemented with $10 \mu\text{M}$ BA or *mT* or TDZ and combinations of $10 \mu\text{M}$ *mT* plus $2 \mu\text{M}$ IAA or NAA at $25 \pm 2^\circ\text{C}$ with a 16-h photoperiod for shoot regeneration. Stored and non-stored synthetic seeds from PGRs-free MS medium were used as controls. The experiments were conducted under aseptic conditions.

Rooting and acclimatization

Shoots (> 2 cm long) obtained via organogenesis and synthetic seeds were cultured onto rooting medium containing semi-solid MS medium with 30 g L^{-1} sucrose and 2.5 – $5 \mu\text{M}$ IAA or IBA or NAA or PG and combinations of $5 \mu\text{M}$ IBA plus $2.5 \mu\text{M}$ IAA or NAA or PG for 10 weeks, as indicated in Table 3. Media devoid of PGRs were used as controls. The plantlets were removed gently from rooting medium after 10 weeks and transferred to terracotta pots (95×120 mm) containing a 1:1 (v/v) vermiculite:soil mixture and irrigated with tap water every third day. These plantlets were maintained in the greenhouse ($25 \pm 2^\circ\text{C}$ under natural photoperiod conditions and a midday PPF of $950 \pm 50 \mu\text{mol m}^{-2} \text{s}^{-1}$) for acclimatization ex vitro.

For all experiments, the chemicals or PGRs used were of analytical grade (Biolab, South Africa; Oxoid, England, and Sigma, USA). All media were adjusted to pH 5.8 with 0.1 N NaOH before gelling with 8 g L^{-1} agar and autoclaving at 121°C for 20 min. The cultures were maintained under 16-h photoperiod supplied by cool white fluorescent light [$40 \mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetic photon flux (PPF), OSRAM L 58 W/740, South Africa] at $25 \pm 2^\circ\text{C}$.

Statistical analysis

All experiments were repeated thrice with 25 replicates for each treatment for shoot regeneration and rooting. Data were collected after 12 and 10 weeks for shoot regeneration and rooting experiments, respectively. The data were subjected to one-way analysis of variance (ANOVA) using SPSS version 24.0 for Windows (Chicago, IL, USA). Significantly

different means were separated using Duncan's multiple range test ($P = 0.05$).

Results and discussion

Direct and indirect adventitious shoot regeneration

Adventitious shoot regeneration was achieved directly from leaf explants and produced different morphogenic responses in vitro on semi-solid MS medium containing 30 g L⁻¹ sucrose, 8 g L⁻¹ agar and different PGRs (Table 1). Shoot

buds were initiated at wounding sites of leaf explants except the control after 4 weeks of culture (Fig. 1a). The shoot-sprouting frequency and morphogenic response (shoot and root regeneration and basal callus) varied with media treatments after 12 weeks (Table 1 and Fig. 1b, c). Combinations of BA or mT or TDZ and IAA or IBA or NAA improved shoot-sprouting frequency (100%) and number of shoots in this plant (Table 1), indicating cytokinin–auxin synergistic effects in shoot proliferation. Similar effects have also been reported in other Hyacinthaceae species (McCartan and Van Staden 1999; Nasircilar et al. 2011; Baskaran et al. 2013; Verma et al. 2016). In contrast, the beneficiary effect

Table 1 Effect of plant growth regulators on direct and indirect in vitro regeneration from leaf explants of *Urginea altissima*

Type of in vitro culture	Plant growth regulators (μM)	Shoot sprouting (%)	Shoots per explant	Shoot length (cm)	Morphogenesis in vitro
Direct regeneration from leaf explant	MS (control)	0	0	0	Explant remained green
	10 BA	50.6 d	4.2 ± 0.26 ef	3.7 ± 0.12 de	SP
	10 mT	65.0 c	2.8 ± 0.30 gh	4.5 ± 0.26 c	SP
	10 TDZ	67.2 b	3.2 ± 0.34 fg	5.6 ± 0.14 b	SP
	10 BA + 2 IAA	100 a	5.8 ± 0.42 cd	4.0 ± 0.36 cd	SP + BC ⁺
	10 BA + 2 IBA	100 a	6.4 ± 0.22 c	3.0 ± 0.18 ef	SP + BC ⁺⁺
	10 BA + 2 NAA	100 a	5.2 ± 0.16 cd	3.9 ± 0.12 de	SP + BC ⁺⁺ + rooting ⁺
	10 BA + 2 PG	100 a	3.6 ± 0.28 fg	2.7 ± 0.20 fg	SP + BC ⁺
	10 mT + 2 IAA	100 a	5.0 ± 0.46 de	3.2 ± 0.32 ef	SP
	10 mT + 2 IBA	100 a	6.1 ± 0.32 c	5.8 ± 0.42 b	SP
	10 mT + 2 NAA	100 a	9.8 ± 0.42 b	7.1 ± 0.38 a	SP + BC ⁺ + thick rooting ⁺⁺
	10 mT + 2 PG	100 a	2.4 ± 0.14 gh	2.0 ± 0.16 g	SP + stunted shoots
	10 TDZ + 2 IAA	100 a	14.6 ± 0.34 a	5.9 ± 0.24 b	SP + BC ⁺
	10 TDZ + 2 IBA	100 a	6.7 ± 0.42 c	3.6 ± 0.30 de	SP + rooting ⁺
	10 TDZ + 2 NAA	100 a	4.5 ± 0.37 ef	4.7 ± 0.46 c	SP
	10 TDZ + 2 PG	100 a	6.3 ± 0.32 c	3.4 ± 0.28 de	SP
Indirect regeneration from callus	MS (control)	0	0	0	No SR + WCC remained
	10 BA	73.3 i	6.2 ± 0.28 de	2.5 ± 0.16 de	SR + YGFC
	10 mT	100 a	9.7 ± 0.32 b	3.8 ± 0.28 cd	SR + GFC + rooting ⁺⁺
	10 TDZ	100 a	4.5 ± 0.20 gh	2.9 ± 0.14 de	SR + GFC
	10 BA + 2 mT	100 a	4.0 ± 0.26 hi	2.3 ± 0.32 de	SR + GCC
	10 BA + 2 TDZ	77.8 h	5.6 ± 0.42 ef	2.7 ± 0.24 de	SR + GCC
	10 mT + 2 BA	60.0 k	6.8 ± 0.36 de	3.5 ± 0.42 cd	SR + GCC + rooting ⁺⁺
	10 mT + 2 TDZ	84.7 f	9.4 ± 0.54 b	5.9 ± 0.48 a	SR + GCC
	10 TDZ + 2 BA	91.0 c	5.0 ± 0.30 fg	3.6 ± 0.24 cd	SR + GCC
	10 TDZ + 2 mT	69.6 j	3.8 ± 0.24 i	2.4 ± 0.36 de	SR + GCNC
	10 mT + 2 NAA	87.5 e	7.0 ± 0.48 d	4.2 ± 0.30 b	SR + GFC
	10 mT + 2 IAA	96.4 b	11.6 ± 0.54 a	4.0 ± 0.34 bc	SR + GFC
	10 mT + 2 Pic	82.2 g	8.4 ± 0.38 c	5.4 ± 0.46 a	SR + GFC
	10 mT + 2 2,4-D	89.2 d	7.8 ± 0.42 cd	4.6 ± 0.28 b	SR + GFC

The data were recorded after 12 weeks of culture. Values are mean ± standard error (SE). Means followed by same letters in each column are not significantly different ($P = 0.05$) using Duncan's multiple range test

SP shoot sprouting, SR shoot regeneration, BC basal callus, BC⁺ basal callus with < 1 g/explant, BC⁺⁺ basal callus with > 2 g/explant, Rooting⁺⁺ > 3 roots + > 3 cm long, Rooting⁺ < 2 roots + < 2 cm long, WCC white compact callus, YGFC yellowish green friable callus, GFC green friable callus, GCC green compact callus, GCNC green compact nodular callus

Fig. 1 In vitro regeneration via direct and indirect organogenesis from leaf and ITCL leaf explants of *U. altissima* in semi-solid and liquid culture. **a** Induction of shoot buds from leaf explants on semi-solid MS medium with 10 μ M BA. **b** Formation of adventitious shoots with basal callus in 10 μ M TDZ plus 2 μ M IAA. **c** Shoot regeneration with basal callus in 10 μ M BA plus 2 μ M NAA. **d** Shoot proliferation in 10 μ M TDZ plus 2 μ M IAA. **e** Simultaneous production of shoots and roots in medium with 10 μ M *mT* and 2 μ M NAA.

f Development of shoot buds on regenerated shoots in TDZ and IBA combinations. **g** Induction of shoot buds on green compact callus in *mT* and TDZ combinations. **h** Shoot multiplication with green friable callus in *mT* and IAA combinations. **i** Prolific shoot induction from ITCL leaf explant on liquid MS medium with *mT* and BA combinations. **j** Shoot proliferation on semi-solid MS medium derived from liquid MS medium with *mT* and BA combinations

of kinetin, 2,4-D, NAA and yeast extract in modified MS medium for production of callus and limited number of shoots has been reported in *U. indica* (Jha et al. 1984). The highest number of shoots (14.6 ± 0.34) with 100% shoot-sprouting frequency was recorded after 12 weeks of culture with a combination of 10 μ M TDZ and 2 μ M IAA (Table 1 and Fig. 1d). The superiority of BA or BA and NAA in modified MS medium for production of bulblets has been reported in *U. maritima* (El Grari and Backhaus 1987; Stojakowska 1993). In this study, the shoot length was increased significantly with 10 μ M TDZ but did not differ significantly with combinations of 10 μ M *mT* and 2 μ M IBA or 10 μ M TDZ and 2 μ M IAA (Table 1). However, the highest shoot length (7.1 ± 0.38) was obtained in combination of 10 μ M *mT* and 2 μ M NAA (Table 1). In this study, combinations of BA and IBA or NAA treatments produced more basal callus (> 2 g per explant), while regenerated shoots induced roots and shoot buds in combinations of *mT* plus NAA and TDZ plus IBA, respectively (Table 1 and Fig. 1e, f). This indicated that *mT* and TDZ with specific auxin influenced simultaneous rooting and shooting. Auxin-like activity has been reported for *mT* and TDZ in other Hyacinthaceae species (Baskaran et al. 2012, 2013).

Indirect organogenesis via callus has been beneficial for biochemical, biomedical and biotechnological studies (Koperuncholan and Ahmed John 2012; Fernando et al. 2016). White compact callus from leaf explants induced shoots with different morphogenic callus in all treatments except the controls after 4 weeks of culture (Fig. 1g). Different morphogenic callus, embryogenic and non-embryogenic were reported in *U. indica* (Jha and Sen 1986). In this study, rapid shoot proliferation was noticed with cytokinin–cytokinin or cytokinin–auxin combinations. This indicated that combination of PGR is essential for prolific shoot regeneration in *U. altissima*. However, higher numbers of shoots with green and friable callus were noticed with combinations of 10 μ M *mT* and 2 μ M IAA after 12 weeks of culture (Table 1 and Fig. 1h). Similarly, shoot multiplication rate and morphogenesis in vitro has been reported to be PGR-dependent in other *Urginea* species (Jha et al. 1984; Stojakowska 1993; McCartan and Van Staden 1999). In this study, rooting of shoots was observed in certain treatments, particularly *mT* and a combination of *mT* and BA (Table 1). The results suggested that *mT* alone or in combination with BA is effective in root regeneration of *U. altissima*. A similar response was observed with other plant species (Baskaran et al. 2013).

Adventitious shoot regeneration from liquid cultures and synthetic seeds

Direct adventitious shoot regeneration was achieved with ITCL leaf explants using different PGRs in liquid media (Table 2). These shoots did not exhibit hyperhydricity. Hyperhydricity from shoot cultures has been reported to be influenced by duration and hormonal effects as well as type of liquid culture (Ascough and Fennell 2004; Grzegorzczak-Karolak et al. 2017). On the other hand, shoot cultures via liquid medium have been useful for the evaluation of physiological and biochemical processes in plants, production of biomass and analysis of pharmaceutical compounds and pharmacological studies (Ascough and Fennell 2004; Georgiev et al. 2011; Grzegorzczak-Karolak et al. 2017). In this study, shoot-sprouting frequency varied with treatments while explants necrosed in controls and to a certain extent

in individual treatments (Table 2). However, prolific shoot regeneration was induced rapidly from ITCL leaf explants in combinations of *mT* and BA or TDZ after transfer to semi-solid MS medium (Fig. 1i). This indicated that ITCL leaf explants on combinations of cytokinins are essential for successful shoot proliferation of *U. altissima*. The TCL technique relies on the thin and small size of the explants and has a limited cell number of homogenous tissue. It has been useful for improvement of commercial production of ornamental, pot and horticultural plants (Rout et al. 2006). The highest numbers of shoots (17.4 per ITCL leaf explant) were produced with a combination of 10 *mT* and 2 BA (Table 2 and Fig. 1j). The present study indicated that TCL explants in liquid culture are useful for rapid shoot proliferation of *U. altissima*.

Synthetic seeds from vegetative parts (shoot tips, axillary buds and nodal segments; alternate to somatic embryos) of

Table 2 Effect of plant growth regulators on direct in vitro regeneration from liquid culture and synthetic seeds of *Urginea altissima*

Type of in vitro culture	Plant growth regulators (μM)	Storage (°C)	Photoperiod (h)	Shoot sprouting (%)	Shoots per explant	Shoot length (cm)	Morphogenesis in vitro
Liquid culture	MS (control)	–	16 Light/8 dark	0	0	0	Explant necrosed
	10 BA	–	16 Light/8 dark	47.6 c	6.2 ± 0.24 d	3.7 ± 0.12 c	SP + necrosis
	10 <i>mT</i>	–	16 Light/8 dark	69.2 b	9.0 ± 0.32 c	2.9 ± 0.10 cd	SP + necrosis
	10 TDZ	–	16 Light/8 dark	34.6 d	5.7 ± 0.28 de	5.0 ± 0.26 b	SP + necrosis
	10 <i>mT</i> + 2 BA	–	16 Light/8 dark	100 a	17.4 ± 0.64 a	3.4 ± 0.34 c	SP
	10 <i>mT</i> + 2 TDZ	–	16 Light/8 dark	100 a	11.6 ± 0.42 b	6.5 ± 0.18 a	SP
Synthetic seeds	MS (control)	–	16 Light/8 dark	41.7 pq	1.0 ± 0.00 i	1.9 ± 0.18 g	No SP
		25 ± 2	Dark	52.4 n	1.0 ± 0.00 i	2.6 ± 0.10 ef	No SP
		4	Dark	34.0 r	1.0 ± 0.00 i	1.7 ± 0.24 g	No SP
	10 BA	–	16 Light/8 dark	68.8 i	3.7 ± 0.42 fg	3.8 ± 0.32 de	SP + BC
		25 ± 2	Dark	79.2 e	4.6 ± 0.50 ef	4.7 ± 0.46 cd	SP + BC
		4	Dark	48.0 o	3.4 ± 0.36 fg	3.2 ± 0.54 de	SP + BC
	10 <i>mT</i>	–	16 Light/8 dark	75.4 f	4.2 ± 0.24 ef	4.5 ± 0.38 cd	SP
		25 ± 2	Dark	82.6 c	6.4 ± 0.38 d	5.6 ± 0.42 c	SP
		4	Dark	60.0 m	3.4 ± 0.20 fg	3.0 ± 0.28 ef	SP
	10 TDZ	–	16 Light/8 dark	65.6 k	3.0 ± 0.16 gh	3.9 ± 0.16 de	SP + rooting ⁺
		25 ± 2	Dark	70.2 h	4.0 ± 0.28 ef	5.0 ± 0.24 cd	SP + rooting ⁺
		4	Dark	42.3 p	3.6 ± 0.32 fg	3.5 ± 0.36 de	SP + rooting ⁺
	10 <i>mT</i> + 2 IAA	–	16 Light/8 dark	72.0 g	5.9 ± 0.46 de	4.7 ± 0.24 cd	SP
		25 ± 2	Dark	81.2 d	8.4 ± 0.38 c	6.2 ± 0.32 b	SP
		4	Dark	62.4 l	5.2 ± 0.22 de	3.8 ± 0.18 de	SP
	10 <i>mT</i> + 2 NAA	–	16 Light/8 dark	84.5 b	9.4 ± 0.54 b	5.3 ± 0.24 c	SP + thick rooting ⁺⁺ + BC
		25 ± 2	Dark	91.0 a	12.6 ± 0.32 a	7.4 ± 0.26 a	SP + thick rooting ⁺⁺ + BC
		4	Dark	67.6 ij	8.2 ± 0.26 c	4.9 ± 0.14 cd	SP + thick rooting ⁺⁺ + BC

The data were recorded after 12 weeks of culture. Values are mean ± standard error (SE). Means followed by same letters in each column are not significantly different ($P = 0.05$) using Duncan's multiple range test

SP shoot sprouting, BC basal callus, Rooting⁺ < 2 roots + < 2 cm long, Rooting⁺⁺ > 3 roots + > 3 cm long

the plant and their storage have been an alternative propagation method for ex situ and germplasm conservation, virus-free, genetically identical materials, easy to handle and transport (Nyende et al. 2003; Ray and Bhattacharya 2008; Islam and Bari 2012). In the present study, stored and non-stored synthetic seeds of shoot tips were prepared from SA (3%) and $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (100 mM) for viability testing and shoot regeneration (Table 2 and Fig. 2a). This was the ideal concentration and combination of SA and $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ to obtain the most uniform beads with a hard enough coat for survival as well as viability of synthetic seeds as put forth by Baskaran et al. (2015). Shoot regeneration of synthetic seeds was initiated after 4 weeks of culture (Fig. 2b). The shoot-sprouting frequencies, shoot number and length and morphogenesis in vitro varied with PGR treatments, storage and photoperiod conditions after 12 weeks (Table 2). Among the different PGR treatments, storage and photoperiod conditions, 10 μM mT and 2 μM NAA and storage of $25 \pm 2^\circ\text{C}$ in the dark proved to be the best for shoot-sprouting frequency (91.0%), and it produced significantly higher shoot numbers (12.6) and lengths (7.4 cm) than other treatments (Table 2 and Fig. 2c). These shoots induced thick roots as well as basal callus (Table 2). Also, TDZ treatments of stored and non-stored synthetic seed-produced shoots promoted rooting (Table 2). These results indicate the influence of specific hormonal effects on synthetic seeds of *U. altissima*. Similarly, temperature for storage and PGRs has affected the rate of shoot regeneration for other plants (Nyende et al. 2003; Ray and Bhattacharya 2008; Islam and Bari 2012). The synthetic seeds that were stored at 4°C showed lower shoot-sprouting frequencies than stored and non-stored synthetic

seeds at $25 \pm 2^\circ\text{C}$. Similarly, lower temperature (4°C) with prolonged synthetic seed storage decreased shoot-forming capacity in *Rauvolfia serpentina* (Ray and Bhattacharya 2008). In the present study clearly indicates that viability of synthetic seeds in *U. altissima* is dependent on the storage temperature. Therefore, the PGR treatment and an optimal temperature for synthetic seeds are essential for viable synthetic seeds and successful prolific shoot regeneration after storage.

Rooting and acclimatization ex vitro

Rooting and acclimatization ex vitro are major components of plant conservation. The regenerated shoots were rooted on semi-solid MS medium containing 30 g L^{-1} sucrose and supplemented with different concentrations and combinations of auxins and PG (Table 3). The number of roots, the length of roots and shoots and morphogenesis of roots varied significantly between treatments (Table 2). Combinations of auxins were effective in rooting of shoots in this plant (Table 3). Effectiveness of auxins combination in rooting has also been reported earlier for many medicinal plant species (McCartan and Van Staden 1999; Rout et al. 2006; Baskaran et al. 2013). The highest number of roots (9.8 roots per shoot) was obtained in media containing combinations of 5 μM IBA and 2.5 μM NAA and was green, thick and healthy (Table 3 and Fig. 2d). Promotion of rooting can be explained by low concentration of auxins and vitamins in other *Urgenia* species (Jha and Sen 1986). The root length (7.2 cm) was significantly higher with 5 μM IBA followed by a combination of 5 μM IBA

Fig. 2 In vitro plant regeneration of *U. altissima*. **a** Production of synthetic seeds using shoot tips. **b** Induction of shoots from synthetic seeds in mT and NAA combinations. **c** Shoot proliferation in mT

and NAA combination from stored synthetic seeds at $25 \pm 2^\circ\text{C}$. **d** Rooting of shoots in combinations of 5 μM IBA and 2.5 μM NAA. **e** Acclimatized ex vitro plants in the greenhouse

Table 3 Effect of auxins and phloroglucinol on rooting of shoots of *Urginea altissima*

Plant growth regulators (μM)	Roots per shoot	Root length (cm)	Morphogenesis of root
MS (control)	1.6 ± 0.10 g	2.2 ± 0.18 h	Thin green roots
2.5 IAA	3.4 ± 0.24 ef	4.6 ± 0.24 d	Thin green roots
5 IAA	3.9 ± 0.38 ef	5.4 ± 0.32 c	Thin greenish-white roots
2.5 IBA	5.0 ± 0.32 d	3.8 ± 0.26 ef	Thin greenish-white roots
5 IBA	6.4 ± 0.28 c	7.2 ± 0.42 a	Thin green and hairy roots type
2.5 NAA	4.8 ± 0.12 de	4.7 ± 0.26 d	Thick white and hairy roots type + BC
5 NAA	3.2 ± 0.54 ef	3.7 ± 0.30 ef	Thick white and hairy roots type + BC
2.5 PG	1.0 ± 0.02 g	2.4 ± 0.08 h	Thin white roots
5 PG	3.0 ± 0.26 f	2.7 ± 0.14 gh	Thin white roots
5 IBA + 2.5 IAA	7.6 ± 0.42 b	4.8 ± 0.42 d	Thin green roots
5 IBA + 2.5 NAA	9.8 ± 0.28 a	6.5 ± 0.38 b	Thick green roots
5 IBA + 2.5 PG	5.2 ± 0.36 d	4.0 ± 0.52 de	Thin green roots

The data were recorded after 10 weeks of culture. Values are mean $\pm$ standard error (SE). Means followed by same letters in each column are not significantly different ($P = 0.05$) using Duncan's multiple range test
BC basal callus

and 2.5 μM NAA treatment (Table 3). Successful rooting of shoots was achieved from different treatments for effective hardening of *U. altissima*.

Well-developed plantlets from rooting medium were harvested and transferred to terracotta pots (95 $\times$ 120 mm) containing 1:1 (v/v) vermiculite:soil mixture. The plants were successfully acclimatized in the maintained greenhouse (Fig. 2e) with a 100% survival rate.

Conclusions

This is the first report of in vitro plant regeneration from organogenesis (semi-solid and liquid culture) and synthetic seed production in *U. altissima* using leaf and ITCL leaf explants. The exogenous hormonal actions for shoot regeneration in *U. altissima* were dependent on explant type. Effective plant regeneration from organogenesis and synthetic seeds was influenced markedly by cytokinin–auxin, cytokinin–cytokinin and auxin–auxin combinations. The synthetic seeds of *U. altissima* could be stored at low temperature (4 °C) retaining the possibility of obtaining above sixty percent of shoot sprouting after storage. This would facilitate transportation and exchange of germplasm across international borders. The establishment of protocols for *U. altissima* provides a great potential for large-scale propagation, germplasm conservation and commercial application such as production of pharmaceuticals and ornamental industry.

Acknowledgements The financial support by the University of Kwa-Zulu-Natal, Pietermaritzburg is gratefully acknowledged.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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