



In vitro and ex vivo vegetative propagation and cytokinin profiles of *Sceletium tortuosum* (L.) N. E. Br.: a South African medicinal plant

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Received: 17 September 2020 / Accepted: 27 December 2020 / Published online: 23 January 2021
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Abstract

Sceletium tortuosum is a South African protected species with tremendous value in traditional and modern medicine. The plants' mesembrine-type alkaloids are potential therapeutics for a plethora of psychological, neurological and inflammatory disorders. In our in vitro and ex vivo studies, vegetative propagation and growth of this species were investigated. Cytokinin (CK) profiles were also explored. Shoot multiplication was induced on Murashige and Skoog (MS) medium supplemented with 2.5 µM indole-3-butyric acid (IBA). In vitro-generated shoots were inoculated on MS medium supplemented with 0, 2.5, 5.0 and 10.0 µM IBA or indole-3-acetic acid (IAA). Optimal rooting (55%), mean number of roots (3.80 ± 0.83) and new leaf pairs (4.65 ± 0.67) were achieved by 10.0 µM IBA. After greenhouse acclimatization, 45–90% of plantlets survived. All ex vivo shoot cuttings rooted well (90–100%). The highest mean number of roots (11.20 ± 1.37) and root length (57.18 ± 3.85 mm) were obtained by 5.0 µM IBA. Although spontaneous rooting was observed in both experiments, auxins enhanced multiple growth parameters. Cytokinin analyses of tissue-cultured (auxin-treated) and greenhouse (untreated) plants revealed higher cytokinin levels in vitro. These investigations provide rapid and efficient propagation techniques for *Sceletium tortuosum* which will be valuable to conservationists and pharmaceutical companies.

Key message

Plant tissue culture and cuttings enabled rapid propagation of *Sceletium tortuosum*. Exogenous plant growth regulators were not essential for shoot multiplication, flowering and rooting. Auxins effectively improved growth parameters.

Keywords *Sceletium tortuosum* · Medicinal plant · Vegetative propagation · Tissue culture · Cuttings · Cytokinin analysis

Abbreviations

BAP N⁶-Benzylaminopurine
 BAP7G N⁶-Benzylaminopurine-7-glucoside
 BAP9G N⁶-Benzylaminopurine-9-glucoside

BAPR N⁶-Benzylaminopurine riboside
 BAPR5'MP N⁶-benzylaminopurine-5'-monophosphate
 CK Cytokinin
 cZ *cis*-zeatin
 cZ7G *cis*-zeatin-7-glucoside
 cZ9G *cis*-zeatin-9-glucoside
 cZOG *cis*-zeatin-*O*-glucoside
 cZR *cis*-zeatin riboside
 cZR5'MP *cis*-zeatin riboside-5'-monophosphate
 cZROG *cis*-zeatin-*O*-glucoside riboside
 DHZ Dihydrozeatin
 DHZ7G Dihydrozeatin-7-glucoside
 DHZ9G Dihydrozeatin-9-glucoside
 DHZOG Dihydrozeatin-*O*-glucoside
 DHZR Dihydrozeatin riboside
 DHZR5'MP Dihydrozeatin riboside-5'-monophosphate
 DHZROG Dihydrozeatin-*O*-glucoside riboside
 GMP Greenhouse mother plant
 IAA Indole-3-acetic acid

Communicated by Ali R. Alan.

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IBA	Indole-3-butyric acid
iP	N ⁶ -isopentenyladenine
iP7G	N ⁶ -isopentenyladenine-7-glucoside
iP9G	N ⁶ -isopentenyladenine-9-glucoside
iPR	N ⁶ -isopentenyladenosine
iPR5'MP	N ⁶ -isopentenyladenosine-5'-monophosphate
K	Kinetin
K9G	Kinetin-9-glucoside
KR	Kinetin riboside
MNLP	Mean number of new leaf pairs
MNR	Mean number of roots
MRL	Mean root length
MS medium	Murashige and Skoog (1962) medium
mT	meta-topolin
mT7G	meta-topolin-7-glucoside
mT9G	meta-topolin-9-glucoside
mTR	meta-Topolin riboside
oT	ortho-topolin
oT7G	ortho-topolin-7-glucoside
oT9G	ortho-topolin-9-glucoside
oTR	ortho-topolin riboside
PAR	Photosynthetic active radiation
PGR	Plant growth regulator
pT	para-topolin
pT7G	para-topolin-7-glucoside
pT9G	para-topolin-9-glucoside
pTR	para-topolin riboside
tZ	trans-zeatin
tZ7G	trans-zeatin-7-glucoside
tZ9G	trans-zeatin-9-glucoside
tZOG	trans-zeatin-O-glucoside
tZR	trans-zeatin riboside
tZR5'MP	trans-zeatin riboside-5'-monophosphate
tZROG	trans-zeatin-O-glucoside riboside

Introduction

Sceletium tortuosum (L.) N. E. Br. (Aizoaceae) is a succulent decumbent shrub native to the south-western regions of South Africa (Gericke and Viljoen 2008). It is utilized in traditional medicine to treat a variety of ailments, including pain, insomnia and anxiety. However, it is most-exploited for its mood-enhancing and calming properties (Digby 2005; Laidler 1928; Rood 1994; Thunberg 1795). The mesembrine-type alkaloids responsible for these effects are suitable treatments for anxiety, stress and depression (Elev8™ 2017; Gericke and Van Wyk 2001; Harvey et al. 2011; Murbach et al. 2014; Terburg et al. 2013). They are also recognized as promising therapeutics for neurodegenerative, psychological and inflammatory disorders, ranging from Alzheimer's disease and drug dependencies to human

immunodeficiency virus (HIV) (Bennett et al. 2018; Coetzee et al. 2016; Dimpfel et al. 2018; Kapewangolo et al. 2016; Napoletano et al. 2001).

Unfortunately, a pure commercial source of mesembrine-type alkaloids does not exist, thus researchers and pharmaceutical companies are reliant upon fresh plant material (Elev8™ 2017; Krstenansky 2017; Wild 2015). *Sceletium tortuosum* is now a protected species due to diminished natural populations (Elev8™ 2017; Gericke and Viljoen 2008). Its value and conservation status suggest that rapid and efficient propagation techniques would be a credit to this species and the pharmacological industry.

Vegetative propagation is a much swifter approach than seed propagation, allowing for conservation of genetic diversity within populations or species (Leakey et al. 1994; LeBude and Blazich 2018). It is particularly useful for medicinal plants because chemotypes are selected for pharmacological use based on their yield of phytochemicals (Elev8™ 2017). Non-aseptic techniques such as grafting, budding, layering and cuttings are common and involve the use of plant organs only (LeBude and Blazich 2018). However, through aseptic plant tissue culture, somatic cells, tissues and organs can be utilized (George et al. 2008; Skoog and Miller 1957). This sophisticated and versatile technique can be easily implemented on a large scale for the production of disease-free plants (Hussain et al. 2012).

Plant growth regulators (PGRs), such as auxins, CKs, gibberellins, ethylene and abscisic acid are widely used in tissue culture (Gaspar et al. 1996; George et al. 2008). Plant growth and phytochemical production can be modified through their use (Amoo et al. 2012; Bairu et al. 2011). However, the use of these PGRs must allow for favourable interactions with endogenous hormones to facilitate healthy plant growth (Gaspar et al. 1996).

Auxins and CKs are regarded as the most influential PGRs for in vitro growth and development (Gaspar et al. 1996). They are well-known for their stimulatory effects on root and shoot growth, respectively (Howell et al. 2003; Overvoorde et al. 2010), but these phytohormones play multiple, often overlapping roles in plant growth and development. Both are involved in the induction and development of root and shoot meristems, branching of lateral and aerial organs and the formation of reproductive organs (Aloni et al. 2006a, b; Chandler 2011; D'Aloia et al. 2011; Schaller et al. 2015; Shimizu-Sato et al. 2009; Su et al. 2011). Thus, various growth characteristics may be directly or indirectly affected by an alteration in the level of even one of these phytohormones (Gaspar et al. 1996; Schaller et al. 2015).

This study aims to develop quick and effective vegetative propagation methods for *Sceletium tortuosum*. In vitro and ex vivo techniques are evaluated and the effects of PGRs (auxins) on growth and development are investigated. Auxin effects on endogenous CK profiles are also explored. To the

best of our knowledge, this is the first comparative vegetative propagation study for this species.

Materials and methods

Plant material

Sceletium tortuosum plants were sourced, collected and identified by Dr J. H. de Lange of Oudtshoorn (S 33° 40.883' E 22° 09.749'). A voucher specimen was deposited in the Bews herbarium (NU0089203) at the University of Kwa-Zulu-Natal (UKZN) in Pietermaritzburg. Plants were potted in a 1:1 mixture of vermiculite and soil and maintained in a greenhouse in the botanical garden at UKZN.

In vitro culture initiation

Shoot nodal segments (2–3 cm) were rinsed under running tap water for 1–2 min. Plant material was immersed in sterile distilled water with 2–3 drops of Tween 20 for 20 min. Material was then washed with 1 g/L Benomyl for 40 min, followed by 0.05% streptomycin sulphate for 20 min. After this immersion, plant material was taken to a sterile laminar flow bench and immersed in 20% hydrogen peroxide (H₂O₂) for 20 min, followed by a wash with 70% ethanol for 2 min. Finally, explants were rinsed three times with sterile distilled water.

Murashige and Skoog (1962) (MS) medium (3% sucrose) was prepared and supplemented with 2.5 µM IBA. The pH of the medium was adjusted to 5.8 using dilute sodium hydroxide (NaOH) and hydrochloric acid (HCl) and solidified with 10 g/L OXOID agar no. 3. The medium was dispensed into tissue culture vessels (20 mL each) and autoclaved (121 °C, 15 psi) for 15 min. Surface-sterilized nodal explants were inoculated onto this medium for shoot multiplication throughout the study. Cultures were incubated at 25 ± 2 °C under constant light (Photosynthetic active radiation (PAR) = 9.07 × 10 µmol.m⁻².s⁻¹).

Cytokinin analysis

Shoots of non-flowering auxin-treated (2.5 µM IBA) in vitro plantlets and untreated greenhouse mother plants (GMPs) were harvested. Plant material was ground in liquid nitrogen and freeze-dried. Sample preparation and CK analysis were carried out via the procedure developed by Novák et al. (2008). Briefly, technical triplicates (2 mg per sample) were homogenized and extracted with 1 mL of modified Bielecki buffer (60% methanol, 10% formic acid, 30% water (Hoyerová et al. 2006)) with a cocktail of stable isotope-labelled internal standards (0.25 pmol of CK bases, ribosides and *N*-glucosides, and 0.5 pmol of CK *O*-glucosides

and nucleotides per sample) (Gupta et al. 2019). Extracts were purified with a combination of C18 (1 mL/30 mg) and MCX (1 mL/30 mg) cartridges (Dobrev and Kamínek 2002). Eluates were evaporated until dry and dissolved in 30 µL of 10% methanol. Cytokinin levels were determined using an ultra-high performance liquid chromatography (UHPLC) device (Acquity UPLC® I-class System; Waters, Milford, MA, USA) coupled to a triple quadrupole mass spectrometer with an electrospray interface (Xevo™ TQ-S, Waters, Manchester, UK). Multiple reaction monitoring of [M + H]⁺ and the appropriate product ion allowed for quantification. MassLynx software was used to compare the ratio of CKs and internal standards of known concentration to determine individual CK levels (Novák et al. 2008; Plačková et al. 2017; Svačinová et al. 2012). Detected CKs are reported in pmol/g DW (dry weight).

In vitro rooting

Nodal explants (1–2 cm) were excised from non-flowering plantlets maintained on 2.5 µM IBA. Explants were inoculated onto MS media supplemented with 0, 2.5, 5.0 and 10.0 µM IBA or IAA. Twenty replicates were used per treatment. Cultures were incubated at 25 °C under constant light (PAR 9.07 × 10 µmol.m⁻².s⁻¹) for 21 days.

Acclimatization

All in vitro-generated plantlets were potted in plastic pots (diameter 75 mm, depth 45 mm) containing a 3:2:1 mixture of soil: sand: perlite. Soil and sand were autoclaved prior to addition of sterile perlite. Plantlets were placed in a mist-house for 1 week where they were watered every 6 min for 10–12 h (daylight hours) and once for 5 min at midnight. Thereafter, they were moved to a greenhouse with watering once a day. Plantlets that survived for 10 days in greenhouse conditions were considered to be acclimatized.

Ex vivo rooting

Shoot pieces (3–6 cm) with 1–2 nodes were used. Shoots with small leaf pairs that had newly emerged were allowed. If present, flowers were removed. The cuttings (cut-ends) were pre-treated with auxin solutions for 10 min prior to potting. Treatments applied were 2.5, 5.0 and 10.0 µM IBA or IAA. The positive control was a pre-treatment in tap water for 10 min while the negative control was untreated. Ten replicates were used per treatment.

Cuttings were planted in plastic pots (diameter 75 mm, depth 45 mm) containing a 3:2:1 mix of sterile soil: sand: perlite and watered with the respective solutions. Both controls were watered with tap water. All cuttings were placed in a greenhouse partially covered with a mesh shade cloth

where they were watered 2–3 times a week (when drying was observed) with the treatment solutions or tap water. Temperature ranged from 7 to 40 °C. Growth was allowed for 21 days.

Measurements and statistical analyses

In vitro and ex vivo rooting experiments were repeated twice. The number of new leaf pairs and roots were recorded manually. Root length (mm) was measured using digital electronic Vernier calipers (model DC001). Root type was also recorded for the in vitro experiment. Roots emanating from callus tissue were classified as non-functional.

All data were analysed using Genstat 18.0. One-way analyses of variance (ANOVA) were performed with Duncan's multiple range tests to identify significant treatment effects on growth parameters. An unbalanced regression analysis was used to determine if treatment and root type had an effect on the number of new leaf pairs. Graphs were generated using GraphPad Prism 8.0.

Results

In vitro cultures and cytokinin analysis

Healthy plantlets with multiple shoots, roots, and in some cases, flowers were generated on MS medium supplemented with 2.5 μ M IBA (Fig. 1). Flower induction was observed in vitro ~ 1–2 months before it was observed in greenhouse mother plants (hereafter referred to as in situ).

The induction of these growth responses (Fig. 1) on auxin-only medium suggested that endogenous CK levels were high. The CK analysis revealed the total CK pool to be three-fold higher in tissue cultured plantlets as compared to in situ plants (Table 1). Aromatic CKs were responsible for 74.7% of the in vitro CK pool, while isoprenoid and aromatic CK pools were approximately equal in situ. Active free CK bases (*cis*-zeatin (*cZ*), *trans*-zeatin (*tZ*) and N^6 -isopentenyladenine (*iP*)) resulted in a larger pool in situ (35.60 pmol/g) than in vitro (27.03 pmol/g). In both samples, *para*-topolin (*pT*) represented the most abundant free CK base (547.33 ± 183.95 and 636.86 ± 132.44 pmol/g for in situ and in vitro shoots, respectively). The four 7-glucosides present in situ were more prevalent in vitro (*tZ*7G, *iP*7G, DHZ7G and *pT*7G), with the most substantial increase observed for *pT*7G (from 56.05 ± 0.71 pmol/g in situ to 1826.09 ± 69.16 pmol/g in vitro). The in vitro sample also contained an additional 7-glucoside (*cZ*7G) that was not detected in situ. Dihydrozeatin-9-glucoside (DHZ9G) was the only 9-glucoside detected in situ (DHZ9G), but it was more abundant in vitro (32.23 ± 2.69). Additionally, *mT*9G



Fig. 1 *Sceletium tortuosum* plantlet obtained from 2.5 μ M IBA after 1 month in vitro. Scale bar represents 1.0 cm

was detected in vitro (484.71 ± 46.76 pmol/g), but not at all in situ (Table 1).

In vitro rooting

There were no significant differences between treatments with regards to mean rooting percentage, with 25–55% of shoot explants producing functional roots after 21 days (Fig. 2a). Although, statistically significant differences were observed between treatments for the remaining growth parameters (Fig. 2). The mean number of roots (MNR) was found to increase in accordance with increasing concentrations of IBA, with the highest MNR recorded for 10.0 μ M IBA (3.80 ± 0.83). The highest mean number of new leaf pairs (MNLP) was also produced by this treatment (Fig. 2b).

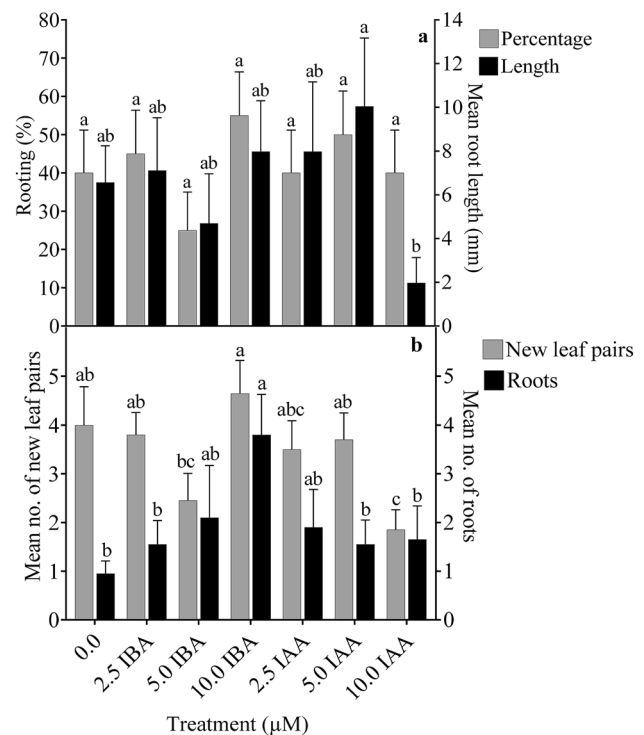
Interestingly, MS media supplemented with 5.0 μ M and 10.0 μ M IAA produced the longest (10.04 ± 3.13 mm) and shortest (1.97 ± 1.16 mm) mean root lengths (MRL) respectively (Fig. 2a). There was no single treatment in which all growth parameters were noticeably low. However, the lowest MNR was recorded in the control treatment (0.95 ± 0.26) (Fig. 2b).

Statistical analysis showed that MNLP was affected by treatment ($P = 0.003$), and by functionality of roots ($P < 0.001$) (Table 2). However, the interaction between treatment and type of roots did not have a significant effect on the number of new leaf pairs produced ($P = 0.608$). Nonetheless, Table 2 shows that plantlets with functional roots have more new leaf pairs than those with non-functional roots.

Table 1 Cytokinins detected (pmol/g DW) in shoots of *S. tortuosum* plants maintained in situ and in vitro (1-month old), shown with total cytokinin pools

Cytokinins detected	In situ	In vitro
cZ	10.55 ± 0.33	15.40 ± 0.33
tZ	<LOD	5.53 ± 0.45
iP	25.05 ± 0.57	6.10 ± 0.32
DHZ	<LOD	<LOD
K	<LOD	<LOD
BAP	<LOD	<LOD
mT	<LOD	<LOD
oT	<LOD	<LOD
pT	547.33 ± 183.95	636.86 ± 132.44
cZR	18.40 ± 2.56	10.20 ± 0.40
tZR	57.36 ± 1.07	38.24 ± 2.24
iPR	268.11 ± 17.76	122.12 ± 1.53
DHZR	7.90 ± 0.02	<LOD
KR	<LOD	<LOD
BAPR	<LOD	<LOD
mTR	<LOD	<LOD
oTR	<LOD	<LOD
pTR	<LOD	<LOD
cZROG	54.34 ± 2.46	209.00 ± 4.08
tZROG	28.20 ± 2.45	26.16 ± 2.35
DHZROG	12.57 ± 0.22	52.35 ± 1.40
cZOG	16.66 ± 1.06	65.07 ± 2.65
tZOG	<LOD	<LOD
DHZOG	4.77 ± 0.20	5.45 ± 0.34
cZ7G	<LOD	153.67 ± 5.52
tZ7G	32.87 ± 3.10	118.25 ± 8.08
iP7G	13.46 ± 0.79	57.40 ± 3.27
DHZ7G	17.61 ± 0.98	80.50 ± 6.71
BAP7G	<LOD	<LOD
mT7G	<LOD	<LOD
oT7G	<LOD	<LOD
pT7G	56.05 ± 0.71	1826.09 ± 69.16
cZ9G	<LOD	<LOD
tZ9G	<LOD	<LOD
iP9G	<LOD	<LOD
DHZ9G	6.33 ± 0.21	32.23 ± 2.69
K9G	<LOD	<LOD
BAP9G	<LOD	<LOD
mT9G	<LOD	484.71 ± 46.76
oT9G	<LOD	<LOD
pT9G	<LOD	<LOD
cZR5'MP	<LOD	<LOD
tZR5'MP	<LOD	<LOD
iPR5'MP	35.36 ± 2.47	<LOD
DHZR5'MP	<LOD	<LOD
BAPR5'MP	<LOD	<LOD
Total isoprenoid cytokinin pool	609.54	997.67
Total aromatic cytokinin pool	603.38	2947.66
Total cytokinin pool	1212.92	3945.33

Results show mean ± SD, <LOD indicates values below the detection limit

**Fig. 2** Rooting percentage and mean root length (a) and mean number of roots (functional) and new leaf pairs (b) of *S. tortuosum* plantlets (n=20) from different treatments after 21 days *in vitro*. Error bars show standard error of the mean. Bars with different letters indicate significant differences between treatments ($P \leq 0.05$)**Table 2** Effect of root type on the mean number of new leaf pairs produced

Type of roots	Mean no. of new leaf pairs ± SE
Functional	5.17 ± 0.42 ^a
Non-functional	2.80 ± 0.20 ^b

Letters superscripts indicate significant differences ($P \leq 0.05$) as per accumulated ANOVA

Acclimatization

Acclimatization was successful for 45–90% of *in vitro*-generated plantlets. The highest percentage of acclimatized plantlets came from the control treatment ($90 \pm 6.9\%$). The best overall treatment for improving growth parameters *in vitro* (10.0 μM IBA) resulted in 50% acclimatization after 10 days (Fig. 4).

Ex vivo cuttings

All treatments and controls resulted in rooted cuttings within 21 days. All but one treatment (10.0 μM IAA) gave

100% rooting (Fig. 5a). The treatment that achieved the highest MNR (11.20 ± 1.37) and MRL (57.18 ± 3.85 mm) was 5.0 μ M IBA (Fig. 5). Only the MNLP was highest in the 2.5 μ M IBA treatment (4.20 ± 0.36) (Fig. 5b). The lowest rooting parameters were found in the positive control (5.20 ± 0.36 and 13.18 ± 1.78 mm for MNR and MRL respectively) (Fig. 5).

Discussion

Cytokinin analysis

A much larger total CK pool was observed in vitro (Table 1), suggesting that auxin played a role in this CK increase. It has been determined that auxin and CK influence and regulate each other's levels (Jones et al. 2010; Jones and Ljung 2011). Although some research has shown auxin to have a negative effect on CK biosynthesis (Jones and Ljung 2011; Nordström et al. 2004; Zhang et al. 1995), our results are consistent with the findings of Bairu et al. (2011). They documented that CK production in *Harpagophytum procumbens* was higher with auxin-only medium compared to the control, and that auxin addition to CK treatments further increased CK levels.

Despite the substantial increase in total CKs in vitro, the majority of these CKs were inactive (*p*T7G and *m*T9G) or minimally active forms (*p*T) (Hothorn et al. 2011; Kamínek et al. 1987). Zhang et al. (1995) documented an increase in adenine derivatives due to enhanced CK oxidase activity caused by auxin. Cytokinin oxidase results in the irreversible inactivation of CKs. However, N-glucosylation can prevent the action of CK oxidase altogether (Kieber and Schaller 2014; Werner et al. 2006). Hence, N-glucosylation may have been triggered to convert CKs to conjugates that can release free CK bases (Hoyerová and Hošek 2020). This reasoning would account for higher levels of 7- and 9-glucosides in vitro (Table 1).

The major metabolic route for CKs in *S. tortuosum* was 7-conjugation (in both samples), though it appears that an additional 9-conjugation pathway was activated in vitro (Table 1). Through this pathway, a previously absent CK conjugate (*m*T9G) was produced in abundance. Despite high levels of this and other inactive CK conjugates, the active free base CK pools of both samples were similar in size (Table 1). This suggests that increased conjugation was induced to regulate the active CK level in response to the large CK quantities being produced (Hoyerová and Hošek 2020). N-glucosylation is a method of inactivating active CKs to achieve said regulation. Furthermore, 7-glucosylation is usually the pathway utilized for quickly modulating active CK levels (Fox et al. 1973; Šmehilová et al. 2016). This mechanism accounts for the heightened levels of

7-glucosides *in vitro*, as well as the higher abundance of *m*T7G rather than *p*T9G (Table 1).

N-glucosides can be metabolized to form their active free bases (Hošek et al. 2020; Hoyerová and Hošek 2020; Mok 2019; Podlešáková et al. 2012), however high levels of 9-glucosides have been shown to cause growth defects and necrosis in vitro (Bairu et al. 2011; Werbrout et al. 1995). Thus, it is noteworthy that no adverse growth effects were observed in *S. tortuosum* (Fig. 1).

The physiological and morphological effects of auxin may have contributed to the enlarged CK pool (Table 1). Auxin stimulates and facilitates cell division and elongation in plants (Cleland 1987; Perrot-Rechenmann 2010). Cytokinin biosynthesis occurs in actively dividing cells, particularly in root and shoot apical meristems and in young leaves (Chen et al. 1985; Nordström et al. 2004). Thus, exogenous auxin likely increased cell division, inducing CK biosynthesis. Rapid growth and morphogenesis (Fig. 1) could have enhanced this biosynthesis, accounting for the overall increase in CKs (Bairu et al. 2011).

Although it seems likely that auxin was responsible for enhanced CK production in vitro, the conditions, specifically constant light exposure, light intensity and quality may have also influenced cytokinin metabolism (Strnad 1997; Zürcher and Müller 2016).

Regardless, the elicitation of multiple growth responses on auxin-only medium (Fig. 1) could be a result of (1) auxin driving these developmental changes (Sassi et al. 2014; Yancheva et al. 2003), (2) a suitable active CK level facilitating these changes (D'Aloia et al. 2011; Werner et al. 2001), or (3) favourable auxin-cytokinin interactions between endogenous and exogenous auxins and CKs (Gaspar et al. 1996; Shani et al. 2006).

Nonetheless, *S. tortuosum* in vitro plantlets demonstrated the ability to produce copious amounts of CKs and elicit several healthy growth responses in the absence of exogenous CKs (Table 1). This is remarkable as such responses are typically elicited with the use of CKs, sometimes in combination with other PGRs (Ashraf et al. 2014; Hesami et al. 2019; Jeong and Sivanesan 2015; Lee and Pijut 2017). These results are contrary to previous findings that the absence of exogenous CKs results in declines in shoot growth and overall plantlet health (Koda and Okazawa 1980; Kudikala et al. 2020; Maheshwari and Kumar 2006). To further understand phytohormone metabolism in *S. tortuosum*, additional PGR treatments and in vitro conditions must be investigated.

In vitro rooting

All auxin treatments successfully generated plantlets with a significant number of new leaf pairs in just 21 days (Fig. 2b). This phenomenon of shoot proliferation in the absence of CKs is not extensively documented. However, Gürel and

Gulşen (1998) noted that shoot growth in two cultivars of *Amygdalus communis* improved with a low concentration of IBA (0.5 μM). Furthermore, Dahab et al. (2005) stated that the number of leaves produced by *Ruscus hypoglossum* increased when half strength MS medium supplemented with 3.0 mg/L IBA ($\approx 15.0 \mu\text{M}$ IBA) was used. These reports support the use of auxin for enhancing shoot proliferation and growth. Additionally, they showed that these parameters were also improved on hormone-free MS media (Dahab et al. 2005; Gürel and Gulşen 1998). This lends support for the high MNLP observed in the control treatment (Fig. 2b).

The ability of auxins to perform their crucial function (root induction) in vitro is well-established (Agarwal and Kamal 2004; Islam et al. 2005; Jahan et al. 2009; Yildirim and Turker 2014), and easily observed in this investigation (Figs. 2 and 3). However, the results indicated that IBA was the more appropriate auxin for rooting nodal explants of *S. tortuosum* (Fig. 2b). This is consistent with other researchers who have achieved greater success with IBA compared to IAA (Islam et al. 2005; Štefančíč et al. 2005). Recently,

Fattorini et al. (2017) reported that IBA controls adventitious rooting in *Arabidopsis thaliana*. Their results showed that 10.0 μM IBA had a stronger effect on root induction than 10.0 μM IAA, which aligns with the findings of this experiment (Fig. 2b).

There was a distinct difference between the lateral growth observed from IBA and IAA treatments. This type of growth is more pronounced in IBA treatments (Fig. 3). Babaei et al. (2014) also reported that lateral root production from adventitious roots increased with the use of IBA in *Curculigo latifolia* cultures.

Despite various studies documenting the potent effects of IBA in vitro, a “carry-over” effect may be partly responsible for the outcomes of the 10.0 μM treatment. This may be explained by the following acclimation hypothesis: multiplication plantlets were grown on media supplemented with 2.5 μM IBA. During the growth period, plantlets may have acclimated to the uptake of this low concentration of IBA. This could have allowed the explants to tolerate and make full use of an even higher concentration during

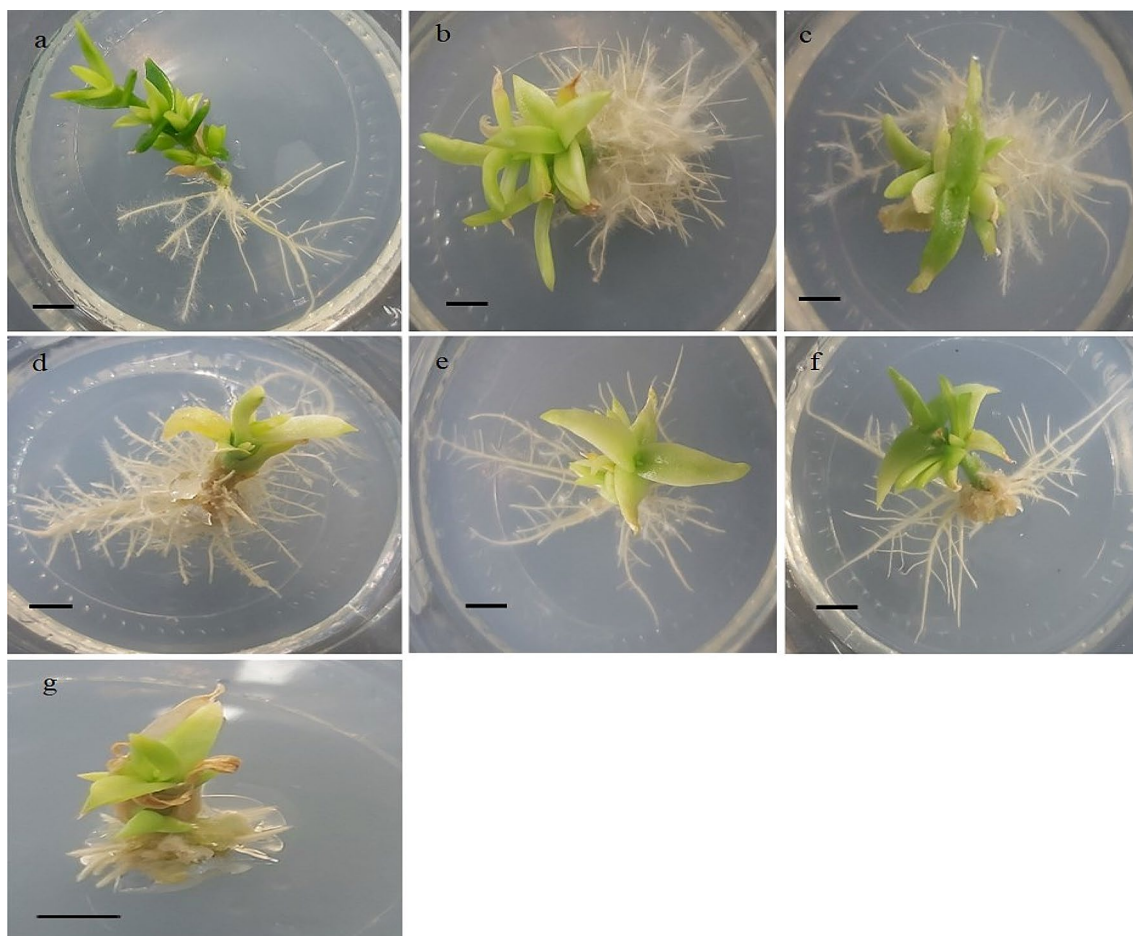


Fig. 3 Rooted in vitro *S. tortuosum* plantlets obtained from different treatments after 21 days of incubation. Treatments correspond to: **a** Control (MS), **b** 2.5 μM IBA, **c** 5.0 μM IBA, **d** 10.0 μM IBA, **e** 2.5 μM IAA, **f** 5.0 μM IAA and **g** 10.0 μM IAA. Scale bar represents 1.0 cm

the experiment (10.0 μM). Following this logic, plantlets had never before experienced an exogenous supply of IAA and may have begun acclimating to this during the experiment. During this process, signalling for the storage of some IAA, as well as for root proliferation would have been ongoing (Korasick et al. 2013; Ljung et al. 2005; Normanly et al. 2010; Overvoorde et al. 2010). It is possible that this combination of signalling resulted in slower mobilization of IAA for root proliferation (Figs. 2b and 3g).

Alternatively, 10.0 μM may have simply been the optimum concentration of IBA, allowing for rapid uptake and conversion to IAA (Ludwig-Müller et al. 2005). Conversely, the same concentration of IAA may have resulted in a disruption in hormone signalling pathways (Li et al. 2009; Overvoorde et al. 2010; Skoog and Miller 1957), likely requiring some time for regulation to be achieved. Such a disruption would explain the slow root growth observed with 10.0 μM IAA (Figs. 2b and 3g).

Despite producing the lowest MNR, rooting was observed in the control treatment (Figs. 2b and 3a). This has been documented in other Aizoaceae species. *Drosanthemum* spp., *Lampranthus* spp. and *Delosperma cooperi* rooted on PGR-free MS medium (Braun and Winkelmann 2015; Mlungwana 2018). Other reports have documented similar findings (Babaei et al. 2014; Islam et al. 2005; Shahzad et al. 2011). Additionally, shoot proliferation was quite successful in this treatment (Figs. 2b and 3a). Thus, these results most closely correlate with findings from Agarwal and Kamal (2004). They reported successful root and shoot proliferation on hormone-free MS medium.

Acclimatization

All but one treatment (10.0 μM IBA) had a greater percentage acclimatized than the percentage of plantlets with functional roots (Figs. 2a and 4). The major discovery here was that plantlets devoid of functional roots were able to acclimatize to greenhouse conditions. This indicates that *S. tortuosum* plantlets possess the ability to induce ex vitro root proliferation unaided by subsequent PGR application. Furthermore, only 40% of control plantlets had functional roots when transplanted ex vitro, however 90% of plantlets acclimatized successfully (Figs. 2a and 4). Thus, with the absence of an in vitro auxin supply, plantlets demonstrated a heightened ability to induce rooting ex vitro and survive.

It can be said that root proliferation occurs spontaneously in this species. Hence, plantlets should be generated on PGR-free MS media in vitro. However, in instances where rooting parameters need to be enhanced by auxins, plantlets should be allowed more time to acclimatize to mist-house conditions prior to transfer to a greenhouse.

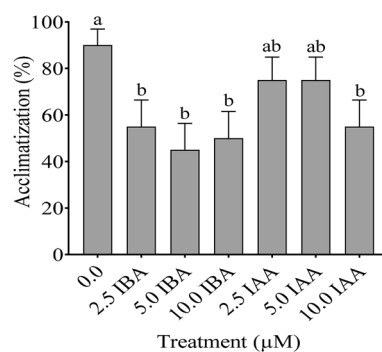


Fig. 4 Acclimatization percentages of in vitro-derived *S. tortuosum* plantlets (n=20) 10 days after transfer to greenhouse conditions. Error bars show standard error of the mean. Bars with different letters indicate significant differences between treatments ($P \leq 0.05$)

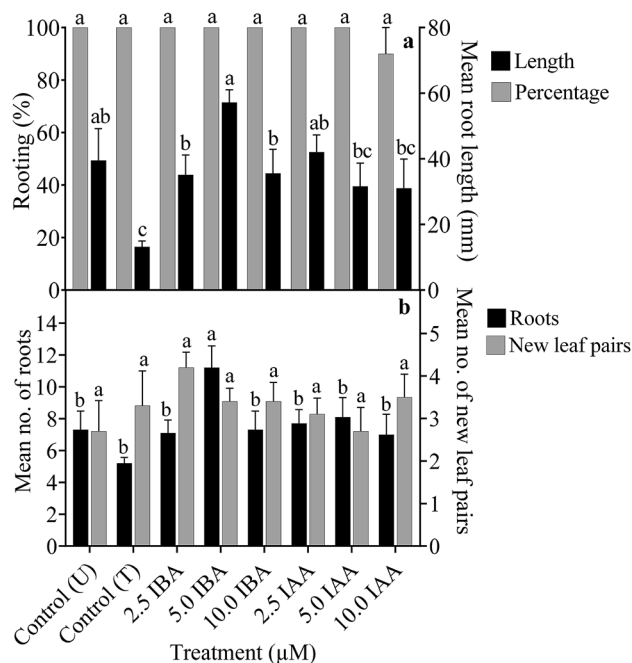


Fig. 5 Rooting percentage and mean root length (a) and mean number of roots and new leaf pairs (b) of *S. tortuosum* cuttings (n=10) from different auxin treatments after 21 days ex vivo. Letters for controls are according to: U=untreated; T=treated. Error bars show standard error of the mean. Bars with different letters indicate significant differences between treatments ($P \leq 0.05$)

Ex vivo rooting

Exogenously applied auxins were not essential to induce rooting in *S. tortuosum* cuttings (Fig. 5a). This suggests that GMPs had sufficiently high auxin levels when shoots were excised. Endogenous auxin levels can improve rooting capabilities (Weigel et al. 1984). Nevertheless, an exogenous auxin supply was able to enhance rooting parameters

(Fig. 5). This is supported by Haissig (1974), who stated that auxins are able to enhance root proliferation even in cuttings that root easily. Indole-3-butyric acid was the better auxin for rooting cuttings of *S. tortuosum*, in terms of MNR and MRL (Fig. 5). Kesari et al. (2009) and Sevik and Guney (2013) recorded similar findings with *Pongamia pinnata* and *Melissa officinalis* cuttings.

Outcomes from control treatments indicated that a short pre-treatment in water resulted in decreased rooting responses compared to the negative control (Fig. 5). Yeboah et al. (2009) showed that submerging cut-ends in water caused auxins and nutrients to leach out. They submerged *Vitellaria paradoxa* cuttings in water for 24 h and rooting was inhibited completely.

The relatively high MNLs and the lack of significant differences between treatments (Fig. 5b) may be explained by the disruption of auxin flow when shoots are severed. This can cause new leaf pairs to emerge on previous nodal segments (Aloni et al. 2006a). These nodal segments were also used in the experiment. More likely though, the state of GMPs at the time of the experiment could account for this. These plants were flowering when cuttings were taken. This reproductive phase is normally associated with high endogenous CK levels (Bernier et al. 1990; Corbesier et al. 2003), which could have contributed to MNLs.

Flowering can upregulate other phytohormone levels as well (Baydar and Ülger 1998). Consequently, when cuttings were supplied with the highest concentration of IBA (10.0 μ M), this may have caused a considerable hormonal imbalance (Li et al. 2009; Overvoorde et al. 2010; Shahzad et al. 2011; Skoog and Miller 1957). This may explain the better responses observed from a lower concentration of IBA (5.0 μ M). Alternatively, if the acclimation hypothesis holds true, the lack of exposure of GMPs to an exogenous auxin could have also played a role.

In both experiments, IBA had a compelling influence on the growth of *S. tortuosum*. Comparatively, the ex vivo experiment produced higher rooting percentages, along with plantlets with more roots and new leaf pairs, as well as longer roots (Figs. 2 and 4). This indicates that cuttings result in faster rooting and more vigorous growth as compared to in vitro-derived plantlets. Although, it should be noted that the in vitro method allows for more efficient propagation due to the shoot multiplication stage.

Conclusions

Root and shoot proliferation, elongation, multiplication and flowering of *S. tortuosum* plantlets occurred on auxin-containing medium (2.5 μ M IBA). Shoot nodal explants and cuttings rooted spontaneously in vitro and ex vivo, however auxins enhanced growth parameters of the plantlets.

The optimal concentrations for growth enhancements differed between experiments, however IBA was more effective than IAA. Ex vivo, 5.0 μ M IBA significantly increased mean root number and length, while 10.0 μ M IBA improved mean number of roots, new leaf pairs and rooting percentage in vitro. Cytokinin levels were higher in in vitro auxin-treated plants, rather than untreated greenhouse plants, suggesting that auxin plays a role in CK metabolism, along with the induction and facilitation of several growth responses.

These vegetative propagation strategies can be used to aid conservation efforts of this protected species. They will also be useful to researchers and pharmaceutical companies. Owing to the value of this species in pharmacology, these methods and additional in vitro methods should be investigated to determine their effects on alkaloid content. This could pave the way towards the production of a commercial source of mesembrine-type alkaloids.

Acknowledgements We are indebted to Dr J. H. de Lange for generously donating plant material for this study. This research was supported by an ERDF project entitled “Development of Pre-Applied Research in Nanotechnology and Biotechnology” (No. CZ.02.1.01/0.0/0.0/17_048/0007323).

Authors' contributions A.S, J.F.F and J.V.S conceived the research idea and design. J.V.S was responsible for the distribution of the materials necessary for the study. A.S conducted the vegetative propagation experiments. L.P and K.D performed the UHPLC-MS/MS analysis. A.S performed the subsequent data analysis and writing of the manuscript. J.F.F and J.V.S supervised the research. All authors reviewed and approved the final manuscript.

Funding This research was supported by a European Regional Development Fund project entitled “Development of Pre-Applied Research in Nanotechnology and Biotechnology” (No. CZ.02.1.01/0.0/0.0/17_048/0007323).

Data availability All data are available from the corresponding author on request.

Code availability Software applications used to analyse data and generate figures are disclosed.

Compliance with ethical standards

Conflict of interest The authors have no conflicts of interest to declare.

Consent for Publication The authors grant permission for this work to be published.

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