Plant Cell Tiss Organ Cult
DOI 10.1007/s11240-010-9885-3

ORIGINAL PAPER

Cytokinin-induced organogenesis in *Lessertia* (*Sutherlandia*) *frutescens* L. using hypocotyl and cotyledon explants affects yields of L-canavanine in shoots

Shakira Shaik · Nisha Singh · Ashley Nicholas

Received: 21 September 2010 / Accepted: 14 November 2010
© Springer Science+Business Media B.V. 2010

Abstract A simple protocol for direct shoot organogenesis and plant regeneration in *Lessertia frutescens* using hypocotyl and cotyledon segments is reported. L-canavanine content in the derived shoots is also quantified. Media containing different concentrations and combinations of the cytokinins kinetin (K) and benzyladenine (BA) were tested for shoot induction potential. The best shoot regeneration rate (83%) was obtained from hypocotyl segments cultured in Murashige and Skoog (MS) medium supplemented with 1 mg l^{-1} K; these hypocotyls also produced the largest number of shoots per explant (3.5) and the longest shoots per explant (13.3 mm). The best shoot regeneration rate (46%) using cotyledons as explant material was obtained in MS medium supplemented with 1 mg l^{-1} K and 1 mg l^{-1} BA or with 5 mg l^{-1} K and 0.5 mg l^{-1} BA. The highest number of cotyledon-derived shoots (1.5) was obtained in MS medium containing 2 mg l^{-1} K and 0.5 mg l^{-1} BA, and the longest cotyledon-derived shoots (6.1 mm) were obtained in MS medium containing 1 mg l^{-1} K and 0.5 mg l^{-1} BA. Shoots derived from hypocotyls cultured on media containing 1 mg l^{-1} K contained the highest quantity of L-canavanine (1.42 mg g^{-1}) relative to the control (0.52 mg g^{-1}). Shoots derived from cotyledons cultured on media containing 2 mg l^{-1} K contained the highest quantity of L-canavanine (2.07 mg g^{-1}) compared to the control. Scanning electron microscopy revealed that shoots regenerated directly from the wounded epidermal tissue, although callus formation was observed in most cultures. Young shoot clusters proliferated into healthy adventitious shoots that were subsequently transferred directly onto rooting medium (MS medium containing 4 mg l^{-1} indole-3-butyric acid), eliminating the need for an additional multiplication or elongation phase. The in vitro plants were successfully acclimatized in a growth chamber, achieving an 85% survival rate.

Keywords Cytokinin · Phytocompound · L-canavanine · Scanning electron microscopy · Shoot regeneration

Introduction

Lessertia (*Sutherlandia*) *frutescens* L. is a well-known, widely utilized medicinal species belonging to the large pod-bearing family Fabaceae. This soft-wooded shrub inhabits veldts, disturbed areas and rocky outcrops throughout the dry regions of southern Africa, particularly in the Eastern and Western Cape provinces of South Africa. There are many reports which highlight the anti-oxidant (Tai et al. 2004), stress-relieving (Prevoo et al. 2008), hypoglycaemic (Chadwick et al. 2007) and anti-mutagenic (Reid et al. 2006 properties of *L. frutescens* leaf extracts. These extracts are also reported to inhibit the action of human immunodeficiency virus (HIV) target enzymes (Hartnett et al. 2005) and decrease cancerous cell growth (Stander et al. 2009), which are purported to be the result of the action of L-canavanine. Research in our laboratory has shown that the yields of medicinal phytocompounds are higher in in vitro leaf extracts than in field leaf or seed extracts (Shaik et al. 2010a).

Rout et al. (2000) reported that medicines derived from plant sources are popular worldwide and, therefore utilized as pharmaceuticals on the global scale. The loss of biodiversity and plantations due to deforestation (Rout et al.

S. Shaik · N. Singh (✉) · A. Nicholas
School of Biological and Conservation Sciences,
University of KwaZulu–Natal, Westville Campus,
Private Bag X54001, Durban 4000, South Africa
e-mail: Singhni@ukzn.ac.za

Published online: 02 December 2010

2000) in combination to the demand from both domestic and export markets (Paek et al. 2005) have lead to the utilization of in vitro methods of propagation as tools to meet commercial needs. Modern technology provides the means to genetically modify specific high-yielding clones to generate commercially valuable natural products. However, the successful genetic transformation of plants depends on an important pre-requisite: the establishment of efficient adventitious shoot regeneration systems in which somatic tissues can develop into whole plants. The increasing concern to conserve biodiversity has also resulted in researchers focussing on efficient in vitro plant regeneration systems.

The in vitro regeneration of plantlets in *L. frutescens* from adventitious buds of aseptic nodal explants (Shaik et al. 2010b) and indirectly through callus derived from stem and rachis segments (Dewir et al. 2010) has been reported. However, Direct organogenesis in *L. frutescens* has not been reported, and there is currently a lack of information on the use of hypocotyls and cotyledons as explants for quick and efficient shoot regeneration. The objective of this study, therefore, was to develop an effective protocol for inducing direct shoot organogenesis from hypocotyl and cotyledon explants in *L. frutescens* and to evaluate the phytocompound yields of the derived shoots. A further objective was to acclimatize the regenerated plantlets.

Materials and methods

Plant material

Lessertia frutescens seeds were obtained from Silverhill Seeds and Books, Kenilworth, South Africa in March 2010. The seeds were subjected to mechanical scarification according to the method described by Shaik et al. (2008). Briefly, all seeds were vigorously rubbed for 6–8 s between two sheets of fine-grained sand paper to remove the testa without injuring the embryo (Pérez-García and González-Benito 2006). As an anti-contamination measure, all seeds were surface sterilized in 70% ethanol for 30 s and then rinsed three times with sterile distilled water prior to in vitro germination. The seeds were placed into sterile plastic petri dishes (9 cm) containing 20 ml of 1% agar. All cultures were incubated at 25°C under a 16/8-h (light/dark) photoperiod with light provided by fluorescent lighting at 40 μ mol m^{-2} s^{-1}. After 24 h, hypocotyl and cotyledon segments were aseptically excised for the induction of adventitious shoots.

Culture of hypocotyls and cotyledons

Hypocotyls and cotyledons were cut into 3-mm-long segments and horizontally inoculated onto various culture media consisting of MS basal salt medium (Murashige and Skoog 1962) supplemented with two different cytokinins [kinetin (K), benzyladenine (BA)] in various concentrations and combinations (Tables 1, 2). As controls, hypocotyl and cotyledon segments were inoculated onto plant growth regulator-free MS medium. All media were supplemented with 3% sucrose and 8% agar. The pH of each medium was adjusted to 5.8 with NaOH prior to autoclaving at 121°C and 1.2 kg cm^{-2} for 20 min. All cultures were incubated for 4 weeks at 25°C under a 16/8-h (light/dark) photoperiod (fluorescent lighting, intensity 40 μ mol m^{-2} s^{-1}).

Rooting and acclimatization

After 4 weeks of culture, adventitious shoots measuring 2–3 cm long were aseptically excised and inoculated directly onto rooting medium containing 4 mg l^{-1} indole-

Table 1 The effect of cytokinins on shoot initiation and development from hypocotyl explants of *Lessertia frutescens*

Cytokinin[a] (mg l^{-1})	No. of shoots	Length of shoots	Days to shoot induction	Shoot regeneration %	Percentage of explants forming shoot primordia	Percentage of explants forming callus	Fresh weight (mg)	Dry weight (mg)
K1	3.5 a	13.3 a	10 a	83 a	75 e	46 e	1,362 c	159 a
K1 + BA0.5	1.5 b	7.3 b	8 b	75 b	88 c	21 g	2,191 a	129 d
K1 + BA1	0.5 c,d	2.1 c	6 c	38 d	96 a	50 d	1,333 d	54 f
K2	1.0 b,c	9.8 a,b	8 b	58 c	83 d	17 h	1,542 b	155 b
K2 + BA0.5	0.3 d	2.0 c	8 b	29 e	96 a	42 f	1,016 f	89 e
K2 + BA1	0.4 c,d	1.6 c	6 c	25 f	83 d	71 a	379 i	35 i
K5	1.0 b,c	7.5 b	6 c	58 c	92 b	42 f	1,203 e	130 c
K5 + BA0.5	0.2 d	1.0 c	8 b	17 g	92 b	63 b	484 h	46 g
K5 + BA1	0.2 d	1.0 c	8 b	13 h	83 d	54 c	532 g	41 h

Mean values within columns followed by different lower case letters are significantly different at $P < 0.05$ by Duncan's multiple range test
K kinetin, *BA* benzyladenine. [a] Numbers indicate concentration (mg l^{-1}) of the cytokinin in the medium

Table 2 The effect of cytokinins on shoot initiation and development from cotyledon explants of *L. frutescens*

Cytokinin[a] (mg l^{-1})	No. of shoots	Length of shoots	Days to shoot induction	Shoot regeneration %	Percentage of explants forming shoot primordia	Percentage of explants forming callus	Fresh weight (mg)	Dry weight (mg)
K1	0.5 b,c	1.5 b	16 a	13 e	71 d	0 f	295 i	41 i
K1 + BA0.5	1.0 a,b	6.1 a	12 b	42 b	83 b	17 c	1,234 b	140 a
K1 + BA1	0.6 b,c	3.3 a,b	12 b	46 a	100 a	8 e	1,177 c	119 c
K2	0.2 c	1.7 b	12 b	13 e	42 f	13 d	327 h	56 h
K2 + BA0.5	1.5 a	3.7 a,b	12 b	42 b	83 b	17 c	1,330 a	135 b
K2 + BA1	0.8 a,b,c	2.4 b	12 b	38 c	79 c	8 e	934 e	74 g
K5	0.2 c	2.4 b	12 b	17 d	63 e	25 a	734 g	111 d
K5 + BA0.5	0.7 b,c	3.3 a,b	12 b	46 a	71 d	21 b	877 f	90 f
K5 + BA1	0.3 b,c	1.0 b	12 b	17 d	71 d	17 c	1,072 d	102 e

Mean values within columns followed by different lower case letters are significantly different at $P < 0.05$ by Duncan's multiple range test

[a] Numbers indicate concentration (mg l^{-1}) of the cytokinin in the medium

3-butyric acid (IBA) using the protocol of Shaik et al. (2010b). After a further 4 weeks of culture, rooted plantlets measuring 3–4 cm in height and consisting of about six to eight roots and four to five leaves were washed free of agar before being transferred to small flower pots filled with an autoclaved and cooled mixture of perlite and vermiculite (1:1; v/v). The plants were watered once a week with a nutrient solution of half-strength MS liquid medium. The pots were covered for the first 10 days with transparent plastic bags to maintain high humidity. These bags were then gradually perforated and completely removed after 4 weeks. All pots were maintained under controlled growth chamber conditions, namely, 25 ± 2°C, 40–50% relative humidity, and a 16/8-h (light/dark) photoperiod with light supplied at an intensity of 100 µmol m^{-2} s^{-1} photosynthetic photon flux. Uncovered plants were housed in the growth room thereafter.

Scanning electron microscopy

Explant segments, which were aseptically removed every 2 days, up to 21 days, were fixed in 2.5% (v/v) glutaraldehyde for 24 h followed by alcohol dehydration through a graded ethanol series. Thereafter, the segments were critical point-dried with CO_2 in a Polaron E3000 critical point drier (Quorum Technologies, Sacramento, CA) followed by sputter-coating with gold in a Polaron E5100 sputter coater. Observations were made and recorded using a LEO 1450 scanning electron microscope (SEM; Zeiss, Oberkochen, Germany) with an EHT of 5 kV and a spot size of 200.

L-Canavanine analysis

Since no shoots grew on the plant growth regulator-free medium, in vitro leaves of stock plants [maintained at 25°C, under a 16/8-h (light/dark) photoperiod and light provided by fluorescent lighting at 40 µ mol m^{-2} s^{-1}] were used as a control (Shaik et al. 2010b). Leaf samples of each treatment, including the control, were separately dried at 60°C for 48 h in a laboratory oven followed by grinding into fine powder using a pestle and mortar. A 30-mg aliquot of each sample was extracted in methanol on an orbital shaker for 24 h. Each extract was vacuum filtrated and concentrated in a rotary evaporator (Buchi, Flawil, Swtizerland) under reduced pressure at 54°C. A 1-mg sample of each concentrated extract was sent to the Central Analytical Facility, Stellenbosch University, South Africa for pre-column derivatization and analysis of L-canavanine content. Analyses were carried out in triplicate.

Experimental design and statistical analysis

Experiments were set up in a completely randomized design. Each treatment had three replications with eight explants per replicate. Several growth parameters were assessed, including shoot number, shoot length, days to shoot induction, percentage of shoot regeneration, percentage of shoot primordia, percentage of explants forming callus, fresh weight and dry weight. Dry weight was recorded after drying the material for 48 h at 60°C, followed by cooling in a desiccator for 6 h. Data were subjected to Duncan's multiple range test using the SAS program (ver. 6.12, SAS Institute, Cary, NC).

Results

Morphological observations of shoot regeneration in hypocotyls and cotyledons

Swelling was observed on the hypocotyl and cotyledon segments 3 days after inoculation, (Fig. 1a, e).

Adventitious shoots were observed in all treatments, except for the control, by 16 days. Hypocotyl explants in all experimental treatments formed shoots more rapidly (6–10 days) than their cotyledon counterparts (12–16 days). Cotyledon explants formed white callus after 8 days and eventually turned yellow to brown without developing into shoots in all treatments except for 1 mg l^{-1} K. Callus primarily originated in hypocotyl segments near the root meristem end, while in the cotyledon experiments, it developed at both wounded ends. Stereomicroscopy (Fig. 1b, f) and scanning electron microscopy (Fig. 2a, b) studies revealed that in both explant types, shoots regenerated directly from the wounded epidermal tissue in contact with the medium. In the hypocotyls, the cut end near the root meristem yielded the most shoots, while in the cotyledon segments, most shoots originated from the cut end opposite to the node. Intervening callus production was not associated with regeneration. Healthy, green and well-developed shoots were clearly observable with the naked eye after 16 days of culture (Fig. 1c, g). In addition to distinct shoots, primordial buds, which were too numerous to count, were also observed in all cultures for each explant type. However, only a few of these developed into shoots longer than 10 mm after the culture period (Fig. 1d, h), and the growth of the remaining primordial buds remained arrested.

Effect of cytokinins on adventitious shoot differentiation

Hypocotyl and cotyledon explants in all experimental treatments led to the induction and differentiation of shoots; however, the type, concentration and combination of the cytokinins directly influenced the shoot regeneration response. Hypocotyl and cotyledon segments inoculated on plant growth regulator-free medium yielded no shoots, while the frequency of adventitious shoot regeneration from hypocotyls and cotyledons ranged from 13 to 83% and from 13 to 46%, respectively (Tables 1 and 2). A large number of primordial buds were also observed proliferating among the larger shoots in all explants. The frequency of primordial bud formation ranged from 75 to 96% and from 42 to 100% in hypocotyls and cotyledons, respectively. The frequency of callus formation ranged from 17 to 71% in hypocotyls and from 0 to 25% in cotyledons, but this did not interfere with shoot regeneration.

In the hypocotyl experiments, the highest shoot regeneration (83%) was obtained in medium supplemented with 1 mg l^{-1} K. The largest number (3.5) and longest shoots (13.3 mm) per explant were also derived in this treatment. However, when the K concentration was increased and was present in the medium in combination with increasing BA levels, the shoot number, shoot length and shoot regeneration percentage decreased. Although the explants in the 1 mg l^{-1} K treatment had significantly better shoot regeneration responses, the time required for shoots to be induced in these explants (10 days) was longer than that required in explants of the other treatments (6 days), such as 1 mg l^{-1} K in combination with 1 mg l^{-1} BA, 2 mg l^{-1} K in combination with 1 mg l^{-1} BA, and 5 mg l^{-1} K.

In the cotyledon experiments, the highest shoot regeneration (46%) was obtained in the treatment containing 1 mg l^{-1} K in combination with 1 mg l^{-1} BA, and 5 mg l^{-1} K in combination with 0.5 mg l^{-1} BA. However, explants cultured on 2 mg l^{-1} K in combination with 0.5 mg l^{-1} BA produced the highest number of shoots (1.5) and those cultured on 1 mg l^{-1} K in combination with 0.5 mg l^{-1} BA produced the longest shoots (6.1 mm). At low K concentrations and when K was present in the medium in combination with lower BA levels, the shoot number, shoot length and shoot regeneration percentage increased; in contrast, at higher BA levels, these declined. Similar to the hypocotyl experiments, the time required for shoots to be induced in cotyledon explants in the 1 mg l^{-1} K treatment (16 days) was longer than that in cotyledon explants in all other treatments (12 days).

Scanning electron microscopy

Scanning electron microscopy examination revealed that shoots regenerated directly from the original explant tissues (Fig. 2a, b). Differentiation into adventitious shoots occurred by day 12 post-culture initiation (Fig. 2c), and well-developed shoots formed by day 16 (Fig. 2d) post-culture initiation. Non-morphogenic callus cells were also observed (Fig. 2b) among developing shoots.

Rooting and acclimatization

After 4 weeks in rooting medium (4 mg l^{-1} IBA), 80% of plantlets had formed healthy white roots (Fig. 3a). Rooted plantlets were transferred to a perlite and vermiculite (1:1, v/v) mixture (Fig. 3b) for 4 weeks and then successfully acclimatized in a growth chamber. The survival rate was 85% (Fig. 3c).

L-canavanine analysis

Figure 4 shows the trends in the yield of L-canavanine in shoots derived from the hypocotyls and cotyledons compared to the control. In general, the quantity of L-canavanine ranged from 0.16 to 1.42 mg g^{-1} in the hypocotyl-derived shoots and from 0.12 to 2.07 mg g^{-1} in the cotyledon-derived shoots. The control yielded 0.52 mg g^{-1} L-canavanine. Of the nine treatments, five

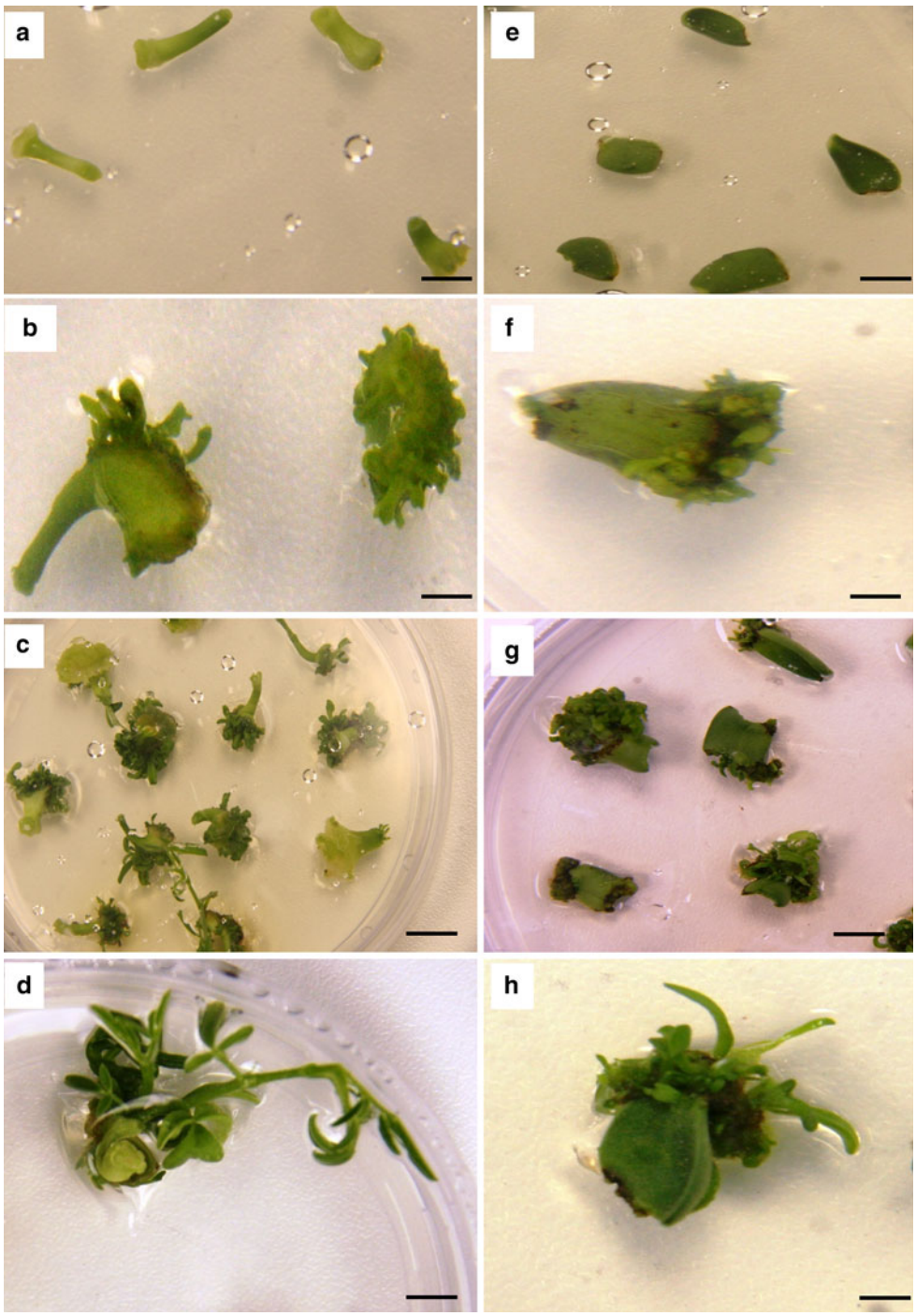

Fig. 1 Hypocotyl segments after 3 days (**a**), 10 days (**b**), 15 days (**c**) and 21 (**d**) days in culture. Cotyledon segments after 3 days (**e**), 10 days (**f**), 15 days (**g**) and 21 days (**h**) in culture. *Bars* 5 mm

yielded significantly higher quantities of L-canavanine in the hypocotyl-derived shoots than the control, namely, 1, 2 and 5 mg l^{-1} K, 1 mg l^{-1} K in combination with 0.5 mg l^{-1} BA, and 2 mg l^{-1} K in combination with 1 mg l^{-1}BA. The highest quantity of L-canavanine (1.42 mg g^{-1}) was found in shoots cultured on media containing 1 mg l^{-1} K. Similarly, of the nine treatments, five yielded significantly higher quantities of L-canavanine in the cotyledon-derived shoots than in the control, namely, 1, 2 and 5 mg l^{-1} K, 2 mg l^{-1} K in combination with 1 mg l^{-1} BA, and 5 mg l^{-1} K in combination with 0.5 mg l^{-1} BA. The highest quantity of L-canavanine (2.07 mg g^{-1}) was found in those shoots (cotyledon-derived), which was also the highest L-canavanine yield overall, cultured on media containing 2 mg l^{-1} K.

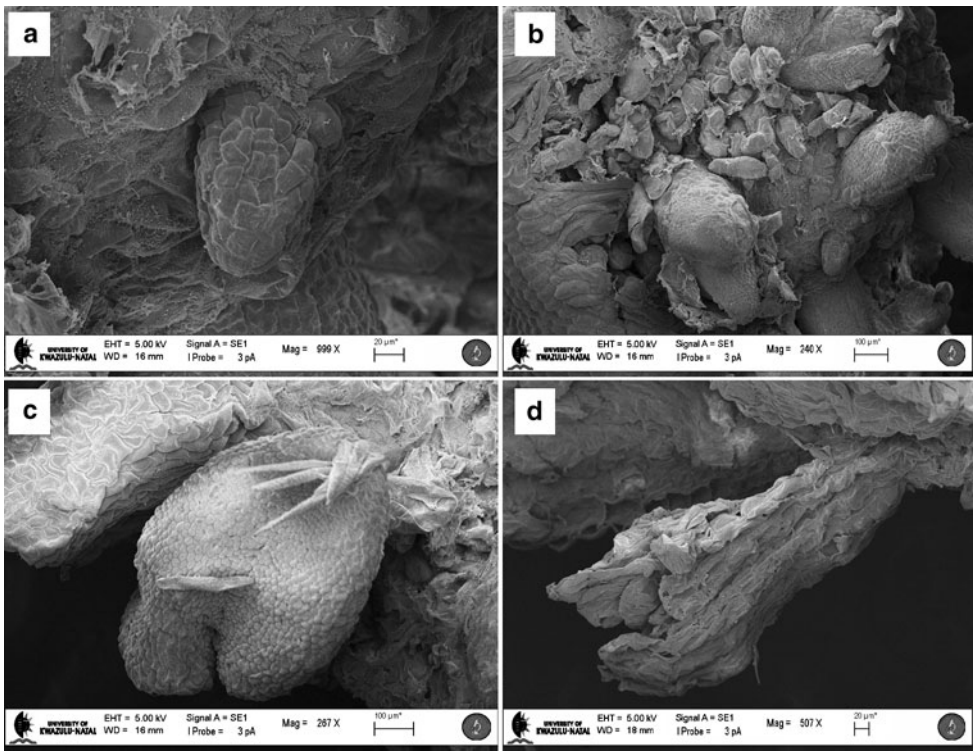

Fig. 2 Scanning electronic microscopy images showing direct shoot organogenesis in hypocotyls after 3 days (**a**) and 8 days (**b**) (note non-morphogenic callus cells between emerging shoots), 12 days (**c**) and 16 days (**d**) in culture

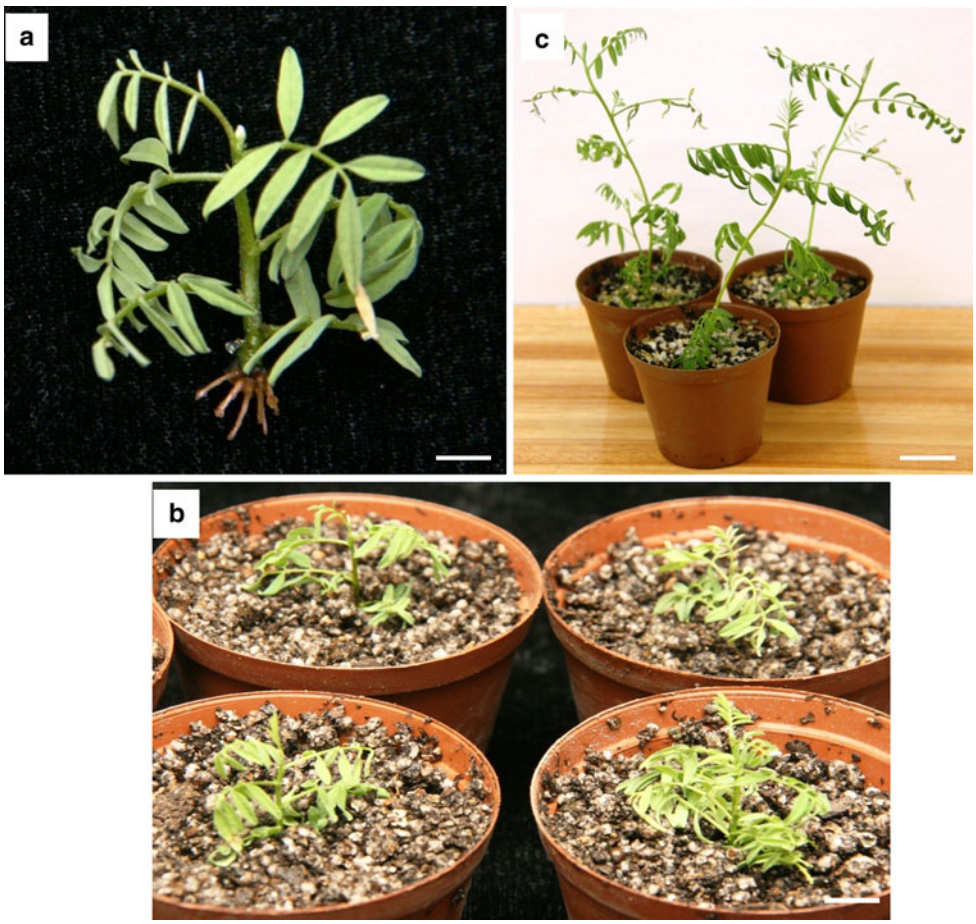

Fig. 3 Rooting and acclimatization in *Lessertia frutescens*. **a** Rooted plantlet after 4 weeks on rooting medium, **b** rooted plantlets in flower pots ready for transfer to the growth chamber, **c** acclimatized plants. *Bars* 5 mm

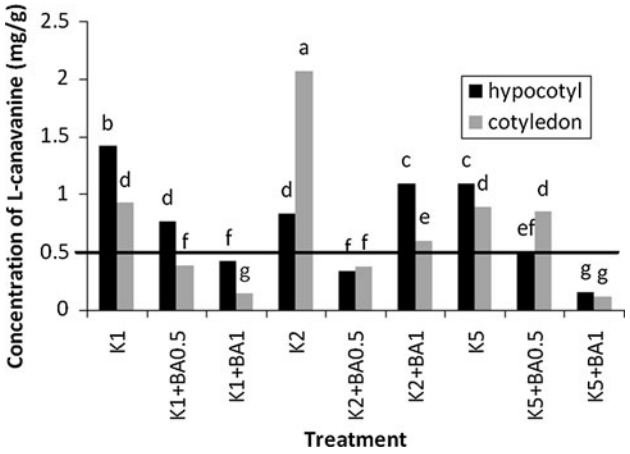

Fig. 4 Concentration of L-canavanine in shoots derived from hypocotyl and cotyledon segments of *L. frutescens* using various treatments. *Solid line* Control value (0.5 mg l^{-1}). *Different letters* (*a–g*) indicate a significant difference between treatments at $P \leq 0.05$ according to Duncan's multiple range test. *K1* 1 mg l^{-1} kinetin, *K1 + BA0.5* 1 mg l^{-1} kinetin + 0.5 mg l^{-1} benzyladenine, *K1 + BA1* 1 mg l^{-1} kinetin + 1 mg l^{-1} benzyladenine, *K2* 2 mg l^{-1} kinetin, *K2 + BA0.5* 2 mg l^{-1} kinetin + 0.5 mg l^{-1} benzyladenine, *K2 + BA1* 2 mg l^{-1} kinetin + 1 mg l^{-1} benzyladenine, *K5* 5 mg l^{-1} kinetin, *K5 + BA0.5* 5 mg l^{-1} kinetin + 0.5 mg l^{-1} benzyladenine, *K5 + BA1* 5 mg l^{-1} kinetin + 1 mg l^{-1} benzyladenine

Discussion

As a general rule, woody plants are difficult to propagate in in vitro culture systems. However, several authors have reported direct plant regeneration from both hypocotyls (Annapurna and Rathore 2010; Nagori and Purohit 2004; Shang et al. 2006; Singh et al. 2002) and cotyledons (Du and Pijut 2008; Raveendar et al. 2009). The data presented in this study clearly demonstrate that high-frequency adventitious shoot regeneration is possible from both hypocotyl and cotyledon segments of *L. frutescens*, also a woody species. Following 4-weeks of culture on plant growth regulator-supplemented media, the adventitious shoots in this study continued to proliferate, showing no signs of senescence or necrosis. Furthermore, the shoots did not require additional transfer to shoot multiplication or elongation medium, which is a standard procedure (Chitra and Padmaja 2005; Kumar et al. 2005; Quintero-Jiménez et al. 2010; Raveendar et al. 2009; Zhang et al. 2008), and were subsequently transferred directly onto rooting medium, thereby eliminating the need for an additional multiplication or elongation phase. In terms of shoot production time, this technique is a more efficient and rapid method of deriving shoots than the indirect organogenesis protocol or the axillary bud culture previously reported (Dewir et al. 2010; Shaik et al. 2010b).

An exogenous supply of cytokinins at varying concentrations and sometimes in combination was a significant factor in promoting shoot induction in *L. frutescens*. K and BA stimulated cell division and growth as a consequence of the plant's inherent plasticity in which the generation of tissues and organs occurs in response to particular stimuli (Rout et al. 2000). The hypocotyls showed a greater regenerative capacity than the cotyledons. Similar findings were reported by Du and Pijut (2008) and Tonon et al. (2001). The distal end of the hypocotyl segment (the section near the root meristem) produced more shoots than the proximal end (the section near the cotyledon), thereby demonstrating a strong polarity action. This difference in regenerative capacity between the two ends may be the result of varying endogenous auxin concentrations in these regions (Lane 1978), which has also been reported more recently by Singh et al. (2002). Shoot regeneration in *L. frutescens* hypocotyls was found to be successful with the application of a single cytokinin, kinetin, at a concentration of 1 mg l^{-1}, even though K is considered to be a relatively weak cytokinin (Koetle et al. 2010). In comparison, optimal shoot regeneration in the cotyledons required relatively higher concentrations of K, both on its own and in combination with BA. This variation in the action of different cytokinins may be a consequence of their differential uptake and translocation rates (Blakesey 1991).

Callus growth was also induced by the application of cytokinins to the medium, but scanning electron microscopy revealed that the callus was of the non-morphogenic type which does not promote secondary differentiation of shoots. The adventitious shoots regenerated directly from the epidermal cells of the original explant tissue without the callus phase, as reported by Shang et al. (2006).

Our analysis of hypocotyl- and cotyledon-derived shoots indicated the presence of the non-protein amino acid, L-canavanine. The exogenous supply of cytokinins, either alone or in combination, and in varying concentrations, was shown to increase the yield of L-canavanine beyond that of the plant growth regulator-free control. However, the origin of the derived shoots, i.e., either from hypocotyls or cotyledons, appears to have had little effect on the increased yields of L-canavanine. This increased production could be attributed to the differential mechanism by which the exogenous cytokinins promote the biosynthesis of L-canavanine either when present individually (Rathore and Shekhawat 2009) or in combination (Goyal and Ramawat 2008). Furthermore, the levels of endogenous L-canavanine, which are determined by chemotype, may have also affected the yields of L-canavanine in this study, as many seeds were used in the initiation of hypocotyl and cotyledon segments.

Our results also reveal that cytokinins in adequate quantities and in suitable combinations as well as the minerals in the media, the carbon sources and culture environment are necessary to increase the yields of the

important phytocompound, L-canavanine (Stafford et al. 1986). These results reflect the importance of plant tissue culture procedures in which the growth medium and conditions can be adjusted to increase yields of important secondary products.

Conclusion

We have developed a rapid and efficient adventitious shoot regeneration, rooting and acclimatization system from hypocotyl and cotyledon segments of *L. frutescens*. The addition of exogenous cytokinins to the medium promoted the initiation and proliferation of shoots and also enhanced the yield of the important medicinal compound, L-canavanine, in this species. The standardization of a highly reproducible protocol that yields high quantities of L-canavanine may be useful in genetic improvement and conservation programmes of this medicinal plant.

Acknowledgements Research funds were made available by the University of KwaZulu–Natal and the National Research Foundation.

References

Annapurna D, Rathore TS (2010) Direct adventitious shoot induction and plant regeneration of *Embelia ribes* Burm F. Plant Cell Tissue Organ Cult 101:269–277

Blakesey D (1991) Uptake and metabolism of 6-benzyladenine in shoot proliferation of *Musa* and *Rhododendron*. Plant Cell Tissue Org 25:69–74

Chadwick WA, Roux S, van de Venter M, Louw J, Oelofsen W (2007) Antidiabetic effects of *Sutherlandia frutescens* in Wistar rats fed a diabetogenic diet. J Ethnopharmacol 109:121–127

Chitra DSV, Padmaja G (2005) Shoot regeneration via direct organogenesis from in vitro derived leaves of mulberry using thidiazuron and 6-benzylaminopurine. Sci Hortic 106:593–602

Dewir YH, Singh N, Shaik S, Nicholas A (2010) Indirect regeneration of the Cancer bush (*Sutherlandia frutescens* L.) and detection of L-canavanine in in vitro plantlets using NMR. In Vitro Cell Dev – Plant 46: 41-46

Du N, Pijut PM (2008) Regeneration of plants from *Fraxinus pennsylvanica* hypocotyls and cotyledons. Sci Hortic 118:74–79

Goyal S, Ramawat KG (2008) Synergistic effect of morphactin on cytokinin-induced production of isoflavanoids in cell cultures of *Pueraria tuberosa* (Roxb. Ex. Willd.) DC. Plant Growth Regul 55:175–181

Hartnett SM, Oosthuizen V, van de Venter M (2005) Anti-HIV activities of organic and aqueous extracts of *Sutherlandia frutescens* and *Lobostemon trigonus*. J Ethnopharmacol 96:113–119

Koetle MJ, Finnie JF, van Staden J (2010) In vitro regeneration in *Dierama erectum* Hilliard. Plant Cell Tissue Organ Cult 103:23–31

Kumar V, Gururaj BC, Narasimha Prasad BC, Giridhar P, Ravishankar GA (2005) Direct shoot organogenesis on shoot apex from seedling explants of *Capsicum annum* L. Sci Hortic 106:237–246

Lane WD (1978) Regeneration of apple plants from shoot meristem tips. Plant Sci Lett 13:281–285

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco cultures. Physiol Plant 15:473–497

Nagori R, Purohit SD (2004) In vitro plantlet regeneration in *Annona squamosa* through direct shoot bud differentiation on hypocotyl segments. Sci Hortic 99:89–98

Paek KY, Chakrabarty D, Hahn EJ (2005) Application of bioreactor system for large scale production of horticultural and medicinal plants. Plant Cell Tissue Org 81:287–300

Pérez-García F, González-Benito ME (2006) Seed germination of five *Helianthemum* species: effect of temperature and presowing treatments. J Arid Environ 65:688–693

Prevoo D, Swart P, Swart AC (2008) The influence of *Sutherlandia frutescens* on adrenal steroidogenic cytochrome P450 enzymes. J Ethnopharmacol 118:118–126

Quintero-Jiménez A, Espinosa-Huerta E, Acosta-Gallegos JA, Guzmán-Maldonado HS, Mora-Avilés MA (2010) Enhanced shoot organogenesis and regeneration in the common bean (*Phaseolus vulgaris* L.). Plant Cell Tissue Organ Cult 102:381–386

Rathore MS, Shekhawat NS (2009) Micropropagation of *Pueraria tuberosa* (Roxb. Ex Willd.) and determination of puerarin content in different tissues. Plant Cell Tissue Organ Cult 99:327–334

Raveendar S, Premkumar A, Sasikumar S, Ignacimuthu S, Agastian P (2009) Development of a rapid highly efficient system of organogenesis in cowpea Vigna unguiculata (L.) Walp. S Afr J Bot 75:17–21

Reid KA, Maes J, van Staden J, De Kimpe N, Mulholland DA, Verschaeve L (2006) Evaluation of the mutagenic and antimutagenic effects of South African plants. J Ethnopharmacol 106:44–50

Rout GG, Samantaray S, Das P (2000) In vitro manipulation and propagation of medicinal plants. Biotechnol Adv 18:91–120

Shaik S, Dewir YH, Singh N, Nicholas A (2008) Influences of presowing seed treatments on germination of the cancer bush (*Sutherlandia frutescens*), a reputed medicinal plant in arid environments. Seed Sci Technol 36:80–795

Shaik S, Singh N, Nicholas A (2010a) HPLC and GC analyses of in vitro-grown leaves of the cancer bush *Lessertia* (*Sutherlandia*) *frutescens* L. reveal higher yields of bioactive compounds. Plant Cell Tiss Organ Cult. doi:10.1007/s11240-010-9884-4

Shaik S, Dewir YH, Singh N, Nicholas A (2010b) Micropropagation and bioreactor studies of the medicinally important plant *Lessertia* (*Sutherlandia*) *frutescens* L. S Afr J Bot 76:180–186

Shang A-Q, Cai H, Yan X-J, Hu H-Z, Zhao L-J (2006) Plant regeneration from in vitro cultured hypocotyl explants of *Euonymus japonicas* Cu zhi. Agric Sci Chin 5:196–201

Singh SK, Meghwal PR, Sharma HC, Singh SP (2002) Direct shoot organogenesis on hypocotyl explants from in vitro germinated seedlings of *Psidium guajava* L. cv. Allahabad Safeda. Scientia Horticulturae 95:213–221

Stafford A, Morris P, Fowler MW (1986) Plant cell biotechnology: a perspective. Enzyme Microb Tech 8:578–587

Stander A, Marais S, Stivaktas V, Vorster C, Albrecht C, Lottering M-L, Joubert AM (2009) In vitro effects of *Sutherlandia frutescens* water extracts on cell numbers, morphology, cell cycle progression and cell death in a tumorigenic and a non-tumorigenic epithelial breast cell line. J Ethnopharmacol 124:45–60

Tai J, Cheung S, Chan E, Hasman D (2004) In vitro culture studies of *Sutherlandia frutescens* on human tumor cell lines. J Ethnopharmacol 93:9–19

Tonon G, Capuana M, Di Marco A (2001) Plant regeneration of *Fraxinus angustifolia* by in vitro shoot organogenesis. Sci Hortic 87:291–301

Zhang Y, Zhou J, Wu T, Cao J (2008) Shoot regeneration and the relationship between organogenic capacity and endogenous hormonal contents in pumpkin. Plant Cell Tissue Organ Cult 93:323–331