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DEVELOPMENTAL BIOLOGY/MORPHOGENESIS

Indirect regeneration of the Cancer bush (*Sutherlandia frutescens* L.) and detection of L-canavanine in *in vitro* plantlets using NMR

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Abstract The present study reports a simple protocol for indirect shoot organogenesis and plant regeneration of *Sutherlandia* using rachis and stem segments. Different concentrations (0.0–68.08 μmol l^{-1}) of thidiazuron (TDZ) were used for callus induction and shoot organogenesis. The highest percentage of callus formation (97.5%) and the highest percentage of explants forming shoots (88.8%) were obtained from rachis explants cultured onto Murashige and Skoog (MS) medium (Murashige and Skoog, Physiol. Plant. 15:473–495, 1962) supplemented with 45.41 μmol l^{-1} TDZ. Scanning electron microscopy demonstrated the early development of adventitious shoots derived from callus cultures. Shoot clusters were further developed and grown in MS hormone-free medium. The presence of L-canavanine was determined by thin-layer chromatography and confirmed after column fractionation using silica gel and nuclear magnetic resonance spectroscopy. Individual shoots were rooted on different concentrations and combinations of MS salt strength and IBA. Half-strength MS salt medium supplemented with 24.6 μmol l^{-1} IBA was optimal for root induction in which 78% of shoots were rooted. The *in vitro* plants were successfully acclimatized in a growth chamber with a 90% survival rate.

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Introduction

Cancer bush (*Sutherlandia frutescens* L.), a member of the Fabaceae family, is a perennial shrub that grows in arid terrains. The plant is native to South Africa, Lesotho, Botswana, and Namibia and has been naturalized in Australia. The plant is a popular ornamental shrub because of its conspicuous scarlet flowers; however, its medicinal benefits render it more widely utilized by traditional healers. It is recorded that British botanists first encountered the cancer bush in South Africa when Zulu traditional healers used it against the influenza pandemic that claimed the lives of approximately 20 million people in the early 1900s (Burcher 2004). Traditionally, the leaves and the aerial parts are used by boiling in water to yield an aqueous infusion used as an astringent tonic (Van Wyk et al. 1997). The plant is a popular remedy with a long history of use for stomach problems and internal cancers (hence the Afrikaans name *kankerbos*), for diabetes, piles, backache, and as a general tonic among the Khoi and Nama people in South Africa (Watt and Breyer-Brandwijk 1962; Van Wyk et al. 1997). In North Africa, it has been used as a bitter tonic and for indigestion and dysentery (Watt and Breyer-Brandwijk 1962). *Sutherlandia frutescens* has recently gained fame in South Africa for its use in patients with HIV/AIDS. It has been documented that it plays a significant role in the treatment of AIDS (Hartnett et al. 2005), cancer (Stander et al. 2007), and diabetes (Chadwick et al. 2007). Its anticancer and antiviral properties have been attributed to the presence of L-canavanine (Van Wyk et al. 1997). It is also effective as an anti-inflammatory

(Kundu et al. 2005), antioxidant (Katerere and Eloff 2005), and anti-mutagenic agent (Reid et al. 2006).

Vegetative propagation of *Sutherlandia* is difficult because the seeds exhibit dormancy which reduces its germination percentage in natural populations (Shaik et al. 2008). Therefore, seedling propagation does not encourage the expansion of the species. *In vitro* methods of propagation are used for the production of medicinal plants to meet the growing demand in both the domestic and the export markets (Paek et al. 2005). In previous reports, cell cultures from *Sutherlandia* were initiated for the production of phytocompounds (Singh and Potukuchi 2006; Smetanska and Riedel 2007). Despite the increasing demand of *Sutherlandia* plants, we could not find any protocols for *in vitro* propagation. The objective of this study was to establish a protocol for inducing shoot organogenesis in *Sutherlandia* using rachis and stem segments. The presence of L-canavanine in *in vitro* plants was investigated by thin-layer chromatography (TLC), column fractionation using silica gel, and nuclear magnetic resonance (NMR) spectroscopy. The present study is crucial for the propagation of *Sutherlandia* since the increasing demand on fresh material as a medicinal herb could result in the attrition of natural populations to the point of extinction and a resultant loss of biodiversity.

Materials and Methods

Plant material and sterilization of explants Sutherlandia. shrubs, 25–35 cm long, were collected from local nurseries in Durban, South Africa. Stem and rachis segments were excised and washed for 10 min under running tap water. Very young or very mature explants were excluded. The explants were then rinsed in 70% (*v*/*v*) ethyl alcohol for 15 s followed by 3% (*v*/*v*) sodium hypochlorite for 5 min. The explants were then washed under sterile conditions with sterile distilled water.

Indirect shoot regeneration. The explants were cut into segments of 0.5 cm and cultured on Murashige and Skoog (MS) medium (Murashige and Skoog 1962) supplemented with 3% sucrose and various concentrations of thidiazuron (TDZ), a substituted phenylurea (*N*-phenyl-1,2,3-thidiazol-5-ylurea), at 0.0, 0.45, 2.27, 4.54, 9.08, 13.61, 22.71, 31.77, 45.41, and 68.08 μmol l^{-1}. TDZ was added prior to autoclaving of the media, and all media were solidified with 0.8% agar. The pH of the medium was adjusted to 5.8 before autoclaving (at 121°C and 1.2 kg cm^{-2} pressure for 15 min). Explants were cultured in 100×15-mm sterile Petri dishes containing 25 ml medium. There were ten explants per Petri dish and four replicates per treatment. Cultures were maintained in light at 40 μmol m^{-2} s^{-1} photosynthetic photon flux (PPF). The percentage of responded explants (those that became swollen and grew), callus formations, and shoot regenerations were recorded after 6 wk of culture.

Scanning electron microscopy. Explants were fixed in 2.5 % (*v*/*v*) glutaraldehyde for 24 h, dehydrated using a graded ethanol series, and critical point-dried with CO_2 in a Polaron E3000 critical point drier. The dried samples were sputter-coated with gold in a Polaron E5100 sputter coater. Observations were made using the LEO 1450 scanning electron microscope.

Development of shoot growth and in vitro rooting. Shoot clumps of *Sutherlandia* were separated and cultured on MS medium without hormones for their subsequent growth. The cultures were kept at 25°C and 40 μmol m^{-2} s^{-1} PPF (16 h/d) for 4 wk. Two- to 3-cm-long shoots were used for rooting in different strengths of MS basal medium (half and quarter strength) supplemented with different concentrations of IBA at 0, 24.6, 49.2, and 73.8 μmol l^{-1}. IBA was added prior to autoclaving of the media and all media solidified with 0.8% agar. The pH of the medium was adjusted to 5.8 before autoclaving (at 121°C and 1.2 kg cm^{-2} pressure for 15 min). The cultures were kept at 25°C and 40 μmol m^{-2} s^{-1} PPF (16 h/d) for 4 wk.

Thin-layer chromatography. Extracts were prepared by grinding 1 g fresh weight of *in vitro* plants using a pestle and mortar and extracted by different treatments (boiled for 5 min in 20 ml sterile distilled water in water bath, soaked in 20 ml ethanol for 1 h, and soaked in 20 ml methanol for 1 h). All extracts were vacuum-filtrated through Whatman no. 4 filter paper and then concentrated in a rotary evaporator under reduced pressure at 54°C. Using a fine glass capillary tube, exactly five drops of Extracts A-H were spotted on pre-coated aluminum plates, silica gel 60 F_{254} (Merck)®, and run in a solvent system comprising *n*-butanol, acetone, ammonium hydroxide, hexane, and water (37:37:19:7). The L-canavanine (1 mg dissolved in 1 ml sterile distilled water) standard was included. The solvent front was pencilled before drying the plate. The air-dried plate was sprayed with 0.2% ninhydrin in acetone (Dr. J. Howard, Sigma-Aldrich Technical Service, St. Louis, MO, e-mail communication). The spots became visible as orange colorations after drying the plate in a laboratory oven at 110°C for 5 min. The developed plate was immediately photographed using a Sony digital Cyber-shot camera.

Column chromatography and NMR. Dried powered leaves, 50 g (DW), were macerated twice at room temperature in methanol for 48 h each and then filtered. The filtrate was

concentrated in a rotary evaporator under reduced pressure at 54°C. The extract was then dried at room temperature in a hood for 5–7 d. The extract was analyzed by TLC where the presence of L-canavanine was indicated. Five hundred milligrams of the dried extract was subjected to column chromatography using a glass column (40×2 cm) filled with approximately 20 g Merck silica gel 60 (0.04–0.063 mm) and eluted with a gradient of *n*-butanol, acetone, ammonium hydroxide, and water (37:37:19:7). Seventy-two fractions of 2 ml each were collected and subjected to TLC. Following further TLC analysis, nine fractions were selected for NMR analysis and were left to dry at room temperature. Approximately 10 mg of dried sample was dissolved in 0.5 ml of deuterated water (d_2O), and the NMR spectra were recorded on a Bruker Advance III 400-MHz instrument.

Acclimatization. Plants at the three- to five-leaf stage were transplanted into culture pots (coffee cups) filled with a mixture of sterilized peat moss and perlite (1:1). The plants were covered with a plastic film during the first 10 d of culture in the growth chamber and watered with a nutrient solution containing half-strength MS salt (Murashige and Skoog 1962). The environment in the growth chamber was adjusted to a 25±2°C air temperature, 40–50% relative humidity, and a 100 μmol m^{-2} s^{-1} PPF with a 16-h photoperiod using halide lamps.

Experimental design and data analysis. Experiments were set up in a completely randomized design and repeated twice. Data were subjected to Duncan's multiple range test using SAS program (version 6.12, SAS Institute Inc., Cary, NC).

Results and Discussion

Stem and rachis segments were cultured on MS medium supplemented with various concentrations of TDZ for

Table 1. Effect of explant type and TDZ concentrations on indirect shoot organogenesis of *S. frutescens* after 6 wk in culture

Explant type	TDZ conc. (μmoll^{-1})	Responded explants (%)	Explants forming callus (%)	Explants forming shoots (%)
Rachis	0.0	25 d	18.8 d	0.0 e
	0.45	72.5 c	72.5 c	12.5 de
	2.27	91.3 ab	91.3 ab	63.8 bc
	4.54	93.8 ab	93.8 ab	71.3 abc
	9.08	94.5 ab	91.5 ab	56.3 c
	13.61	93.8 ab	93.8 ab	80.0 abc
	22.71	97.5 a	97.5 a	60.0 c
	31.77	98.0 a	98.0 a	77.5 abc
	45.41	97.5 a	97.5 a	88.8 a
	68.08	97.5 a	97.5 a	83.8 ab
Stem	0.0	0.0 e	0.0 e	0.0 e
	0.45	30 d	30.0 d	10.0 de
	2.27	80.0 bc	80.0 c	10.0 de
	4.54	92.5 ab	92.5 ab	20.0 de
	9.08	87.5 ab	87.5 ab	20.0 de
	13.61	92.5 ab	92.5 ab	20.0 de
	22.71	95.0 ab	95.0 a	32.5 d
	31.77	87.5 ab	87.5 ab	27.5 d
	45.41	92.5 ab	92.5 ab	12.5 de
	68.08	92.5 ab	92.5 ab	0.0 e
Significance[y]				
Explant type (ET)		—[y]	—[y]	—[y]
TDZ conc. (TDZC)		—[y]	—[y]	—[y]
ET × TDZC		—[y]	—[y]	—[y]

[z] Mean separation within *columns* by Duncan's multiple range test at 5% level

[y] Significant at $P \leq 0.001$

callus induction and shoot formation (Table 1). Rachis segments cultured on MS hormone-free medium formed calluses (18.8%), but these calluses failed to regenerate shoots. Stem segments showed no response on MS hormone-free medium, and TDZ was a prerequisite for their callus induction. The percentage of callus formation significantly increased at all media supplemented with TDZ compared to the control (hormone-free MS medium). Callus formation also increased with increasing TDZ concentrations. The highest percentage of callus formation from rachis segments was observed on media containing 22.71 up to 68.08 μmol l^{-1} TDZ, while the highest percentage of callus formation from stem segments was observed on media containing 4.54 up to 68.08 μmol l^{-1} TDZ. The proliferation responses were influenced by TDZ concentration and the explant type. The highest shoot formation percentage (88.8%) was obtained from rachis explants cultured on medium supplemented with 45.41 μmol l^{-1} TDZ, whereas the highest shoot formation percentage from stem segments (32.5%) was obtained from medium supplemented with 22.71 μmol l^{-1} TDZ. The shoot organogenesis percentage from stem segments was significantly lower than that from rachis segments at all the TDZ concentrations tested.

The results obtained indicate that the explant type is a key factor influencing indirect shoot organogenesis in *S. frutescens*. Both direct and indirect shoot regeneration require plant cells to undergo dedifferentiation and redifferentiation, both of which are known to be affected not only by exogenous plant growth regulators but also endogenous content of the hormones (Trigiano and Gray 2000). The type of explant would have a critical impact on the regeneration success. In the present study, it was clear that rachis segments were much more productive for shoot organogenesis than stem segments, producing nearly three times more shoots. The TDZ concentration is also an important factor influencing shoot organogenesis in *Sutherlandia*. TDZ is considered to be one of the most active cytokinins for shoot induction and a potent bioregulant of *in vitro* morphogenesis (Huetteman and Preece 1993; Murthy et al. 1998). TDZ-induced shoot organogenesis from different explants of many recalcitrant species as well as from medicinal plants has been reported (Murthy et al. 1998; Schween and Schwenkel 2002; Liu et al. 2003; Mithila et al. 2003; Thomas 2003). Several reports suggest that TDZ results of shoot regeneration are better than the results from other cytokinins (Barna and Wakhlu 1995; Thomas 2003). TDZ-induced morphogenesis probably depends on the levels of endogenous growth regulators, and TDZ modulates the endogenous auxin levels (Murthy et al. 1995; Hutchinson and Saxena 1996).

Callus formation of *Sutherlandia* was observed within 1 wk of culture (Fig. 1*A*), and nodular calluses were observed after 2 wk of culture (Fig. 1*B*) on MS medium supplemented with 45.41 μmol l^{-1} TDZ. Small green meristems were visible on the surface of calluses after 4 wk of culture (Fig. 1*C*). These meristems were then developed into shoot buds (Fig. 1*D*), and shoot clusters with leaves were developed by the end of 6 wk of culture on the same medium. The regenerated shoots via indirect shoot organogenesis were separated and cultured for 4 wk on hormone-free MS medium for their further growth and development.

The presence of L-canavanine was detected as a bright orange spot following spraying with 0.2% ninhydrin and was evident in all the samples. The Rf value for L-canavanine was 0.25. The early fractions (1–8) following column separation and TLC detection for L-canavanine were chlorophylls and were not further analyzed. Fractions 9–20 contained a mixture of compounds and were therefore not further analyzed. The presence of L-canavanine was confirmed in fractions 21–29 which were then combined and analyzed by NMR. Characterization by NMR confirmed the presence of L-canavanine in the samples following ^{1}H and ^{13}C analyses and possessed identical spectra in comparison with the standard (L-canavanine, Sigma, $C_5H_{12}N_4O_3$).

Developed *Sutherlandia* shoots were cultured in media containing different concentrations and combinations of MS salt strength and IBA for their rooting (Table 2). Root differentiation was observed after about 1 wk in culture. On half-strength MS and quarter-strength MS salts medium without IBA, the rooting percentage was low, only 29.5% and 34%, respectively. The addition of IBA to the culture medium produced the more evident effects. The optimal medium for rooting contained half-strength MS salts and 24.6 μmol l^{-1} IBA on which a maximum of 78% shoots rooted with an average 5.5 roots per shoot and an average root length of 2.1 cm. Complete plants were obtained 4 wk after the regenerated plants were transferred to this medium. A combination of low concentration of IBA (<24.6 μmol l^{-1}) and full-strength salt MS resulted in callus formation and poor rooting; moreover, these roots were thin and easy to cut when washed out of medium (data not shown). Basal medium has an important influence on root induction and root quality (Garland and Stoltz 1981; Zimmerman and Broome 1981). In several tissue cultures of wood species, higher ion strength inhibited adventitious root induction and growth (Manzanera and Pardos 1990; Purohit et al. 1994; Tao et al. 1999). For *Sutherlandia*, we also found that full-strength MS medium inhibited root formation. Half-strength MS medium supplemented with high concentrations of IBA resulted in a satisfactory rooting. The rooted shoots were green and healthy, probably because of a low content of nitrogen ions in the rooting medium. IBA is the most commonly used auxin for root

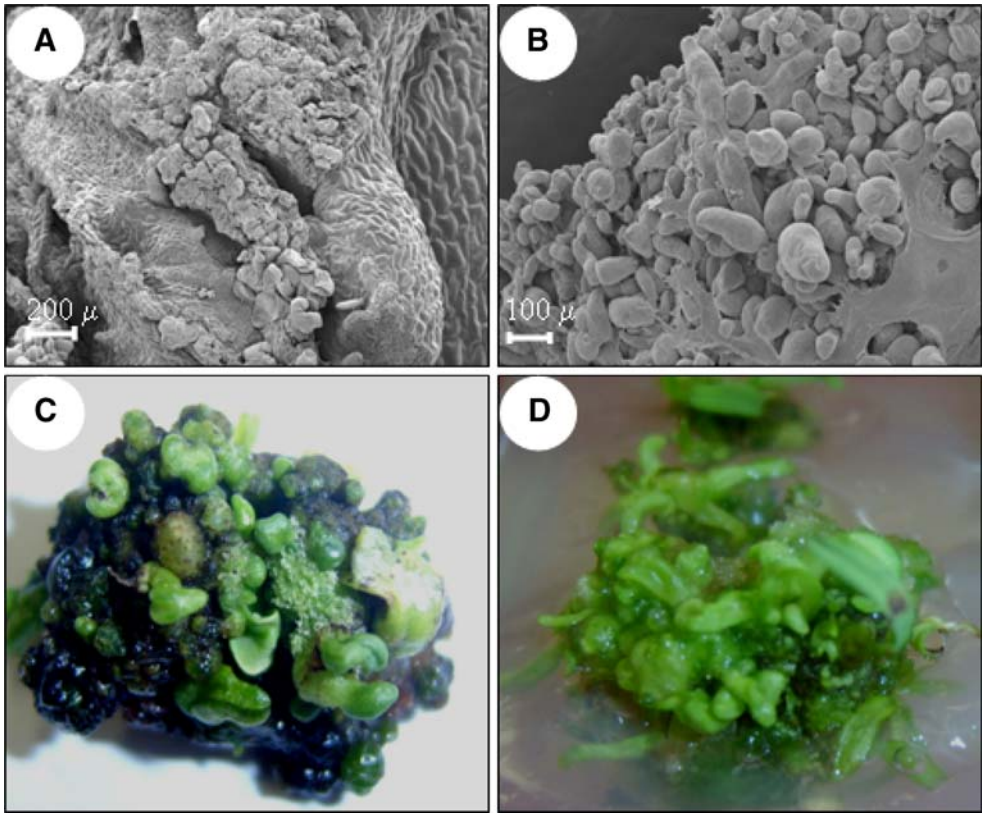

Figure 1. Indirect shoot organogenesis in *Sutherlandia* using rachis segments. *A*, Callus formation after 1 wk of culture. *B*, Nodular meristematic callus after 2 wk in culture. *C*, Greenish organogenic clusters showing shoot primordia after 4 wk in culture. *D*, Cluster of developed shoots after 6 wk in culture.

Table 2. Effect of MS salt strength and IBA concentrations on rooting of *S. frutescens* after 4 wk in culture

MS salt strength	IBA conc. ($\mu moll^{-1}$)	Rooting (%)	No. of roots/ explant	Length of main root/ explant (cm)	Plant length (cm)	No. of leaves/ explant	Fresh weight (mg)	Dry weight (mg)
1/2 strength	0	29.5 d[z]	4.3 bc	1.8 b	3.1 c	4.1 bc	252 bc	41 ab
	24.6	78 a	5.5 ab	2.1 b	5.7 a	6.8 a	365 a	52 a
	49.2	66 ab	6.8 a	2.2 b	4.5 ab	4.9 b	328 ab	45 ab
	73.8	33 d	3.0 cd	3.9 a	2.4 c	2.6 cd	240 bc	38 ab
1/4 strength	0	34 d	1.8 d	0.9 b	2.3 c	3.2 bcd	176 c	36 b
	24.6	55 bc	3.5 cd	1.5 b	3.2 bc	4.1 bc	215 c	38 ab
	49.2	44 cd	3.0 cd	2.0 b	2.6 c	3.1 cd	171 c	33 b
	73.8	33 d	2.3 cd	1.0 b	2.5 c	2.2 d	166 c	35 b
Significance[y]								
Salt strength (SS)		–[x]	–[w]	–[w]	–[w]	–[w]	–[w]	–[x]
IBA concentration (IBAC)		–[w]	–[y]	NS	–[y]	–[w]	NS	NS
SS × IBAC		NS	NS	–[y]	–[y]	NS	NS	NS

NS not significant

[z] Mean separation within columns by Duncan's multiple range test at 5% level

[y] Significant at $P \leq 0.05$

[x] Significant at $P \leq 0.01$

[w] Significant at $P \leq 0.001$

induction. The superior role of IBA in root induction has been reported in several plants (Fracro and Echeverrigaray 2001; Abrie and Van Staden 2001; Thomas 2003). *In vitro* plants of *Sutherlandia* at 4–6 cm in length were then grown in growth chamber, and the plants were acclimatized successfully with a 90% survival rate.

In conclusion, the present study reported a simple protocol for *in vitro* production of *Sutherlandia* plants via indirect shoot organogenesis. To the best of our knowledge, this is the first report on the micropropagation of *L. frutescens*. The findings demonstrate that *in vitro* propagated plants have medicinal properties similar to that in field plantings. The results of this protocol are also useful to satisfy the increasing demand on fresh material of *Sutherlandia* as a medicinal herb and will prevent the attrition of natural populations to the point of extinction and loss of biodiversity. Further studies on optimizing the *in vitro* cultural conditions for the production of L-canavanine and mass propagation of *Sutherlandia* using bioreactor culture system are in process.

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