Journal of Medicinal Plants Research Vol. 5(14), pp. 3078-3083, 18 July, 2011
Available online at http://www.academicjournals.org/JMPR
ISSN 1996-0875 ©2011 Academic Journals

Full Length Research Paper

Micropropagation and detection of important triterpenes in *in vitro* and field grown plants of *Syzygium cordatum*

Yaser Hassan Dewir[1,2]*, Nisha Singh[2], Senabelo Mngomezulu[3] and Ali Mikael Kalifa Omar[4]

[1]Department of Horticulture, Faculty of Agriculture, Kafrelsheikh University, Kafr El-Sheikh 33516, Egypt.
[2]School of Biological and Conservation Sciences, University of KwaZulu-Natal, Private Bag X54001, Durban 4001, South Africa.
[3]School of Chemistry, University of KwaZulu-Natal, Private Bag X54001, Durban 4001, South Africa.
[4]Department of Horticulture, Faculty of Agriculture, Omar Al-Mukhtar University, El-Beida 919, Libya.

Accepted 10 March, 2011

Syzygium cordatum **(Myrtaceae), a recalcitrant tree species, commonly known as 'umdoni'or water berry is known for its medicinal uses. Extracts of the plant are taken as remedies for various ailments including tuberculosis. This study reports on developing a protocol for *in vitro* propagation of *S. cordatum* and the detection of important triterpenes in *in vitro* and field grown plants. A hundred percent seed germination occurred on Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) within 2 to 3 days of culture. *In vitro* developed *Syzigium* shoots were cultured on MS medium supplemented with various concentrations of Thidiazuron (TDZ) and Indole butyric acid (IBA) for their multiplication. MS medium supplemented with 0.5 mg L^{-1} TDZ and 1.0 mg L^{-1} IBA is proved to be most effective in which 4.3 shoots per explants were obtained. The best rooting medium was ½ MS or ½ woody plant medium (WPM) supplemented with 1 mgL^{-1} IBA in which 92% rooting with an average of 3.7 roots per plantlet were obtained. *In vitro* and field leaf materials were oven-dried, grounded into fine powders and extracted sequentially in hexane, dichloromethane, ethyl acetate and methanol. Betulinic acid (BA), oleanolic acid (OA) and ursolic acid (UA) were investigated *in vitro* and field plants by thin-layer chromatography (TLC), column fractionation using silica gel, and nuclear magnetic resonance (NMR) spectroscopy.**

Key words: Betulinic acid, micropropagation, Myrtaceae, oleanolic acid, recalcitrant, triterpenes, Umdoni.

INTRODUCTION

Terpenes are a wide-spread group of natural compounds which comprised of hydrocarbons, derived from isoprene units. Betulinic acid (BA), an important triterpene, and its derivatives have been discovered as a new class of compounds that seem to protect the cells of human immunological system *in vitro* from attack by the HIV virus (Soler et al., 1996). It has been confirmed that BA and also some other triterpenoide acids inhibit HIV 1 replication (Ma et al., 1999). The antiviral properties of BA were also confirmed in clinical trials (De Clercq, 2000). The antitumor-activity of BA is strongly selective against human melanoma cells (Selzer et al., 2000). As is

*Corresponding author. E-mail: ydewir@hotmail.com Tel: +20194046715. Fax: +20479102930.

Abbreviations: BA, Betulinic acid; **DW,** Dry weight; **FW,** Fresh weight; **IBA,** Indole butyric acid; **NMR,** Nuclear magnetic resonance; **OA,** Oleanolic acid; **PGRs,** Plant growth regulators; **PPF,** Photosynthetic photon flux; **TDZ,** Thidiazuron; **TLC,** Thin-layer chromatography; **UA,** Ursolic acid.

generally acknowledged, BA induces apoptosis that is the controlled death of a cancer cell. Being selective towards melanoma cells, it does not affect normal cells (Zuco et al., 2002). This feature makes BA unique in comparison to compounds that are currently used in cancer therapy, such as taxol, camptothecin, elipticine, etoposide, vinblastine or vincristine.

Syzygium cordatum Hochst.ex C.Krauss (Myrtaceae) commonly known as umdoni or water berry, is an evergreen tree which grows to a height of 8 to 15 m and known for its many uses. As a medicine, extracts of the plant are taken as remedies for stomachache, diarrhoea, respiratory ailments, tuberculosis, mild diabetes mellitus and glucose intolerance (van Wyk et al., 1997; van Wyk and Nigel, 2000; Musabayane et al., 2005). Antibacterial properties of *S. cordatum* extracts against *Vibro cholera*, *Escherichia coli, Staphylococcus aureus*, *Shigella* spp., and *Salmonella typhi* have been demonstrated (Mathabe et al., 2006). Candy et al., (1968) reported on the wood and bark constituents; however, there are no recent phytochemical studies on this species. The seeds of *S. cordatum* are recalcitrant and can not tolerate dehydration below high critical water content and therefore germplasm storage is problematic (Cheruiyot et al., 2004). *In vitro* plant propagation methods have been developed for some *Syzygium* species (Mathew and Hariharan, 1990; Yadav et al., 1990; Sha Valli Khan et al., 1997, 1999; Remashree et al., 2007). Tissue culture techniques for the clonal propagation and *in vitro* conservation of these rare and endemic species have proven useful for maintaining its population. However, there have been no reports on *in vitro* propagation of *S. cordatum*.

Therefore, the objective of the present study was to develop a micropropagation protocol for *S. cordatum*, seeing as sexual as well as vegetative propagation methods are beset with many problems which restrict its multiplication on large scale. The presence of BA, oleanolic acid (OA) and ursolic acid (UA) in *in vitro* and field plants was investigated by thin-layer chromatography (TLC), column fractionation using silica gel, and nuclear magnetic resonance (NMR) spectroscopy.

MATERIALS AND METHODS

Plant material and seed germination

Fully ripe fruits of *S. cordatum* Hochst. ex C. Krauss were collected from trees growing at the University of KwaZulu-Natal, Westville, South Africa. To confirm identification, the fruits were referenced against voucher specimen deposited in the University's Ward Herbarium (MH/05). The seeds were manually removed from the fruits, washed and air dried. Small and injured seeds were excluded. The seeds were rinsed in 70% (v/v) ethanol for 30 s followed by 0.15% $HgCl_2$ containing one drop of Tween 80 for 5 min under a constant hand agitation. Seeds were then washed with sterile distilled water and rinsed in 3.5% sodium hypochlorite containing one drop of Tween 80 for 5 min followed by 1.75% sodium hypochlorite for 10 min. Seeds were then washed thrice with sterile distilled water for 5 min each. The seeds were inoculated in Petri dishes each containing 20 ml of MS medium (Murashige and Skoog, 1962) supplemented with 3% (w/v) sucrose and 0.8% (w/v) agar without plant growth regulators (PGRs). The pH of the medium was adjusted to 5.8 before autoclaving for 20 min at 121°C. The cultures were incubated at 25 ± 2°C under dark conditions. After one week of culture, germinated seeds were sub-cultured into 250 ml Majenta (Sigma) plastic vessels containing 50 ml of the same medium for 4 weeks. All cultures were incubated at 25 ± 2°C with 16 h light at 55 µmol m^{-2} s^{-1} photosynthetic photon flux (PPF) provided by cool white fluorescent tubes. Shoot tips and nodes of the proliferated plantlets were maintained on MS medium without PGRs and kept at 25 ± 2°C with 16 h light at 55 µmol m^{-2} s^{-1} PPF. Subsequent sub-cultures for one year gave more shoots until sufficient stock material was produced and used for further experiments.

Shoot multiplication and *in vitro* rooting

In vitro shoots (3 to 4 cm in length) were served as initial plant material for shoot multiplication experiments. Syzygium shoots (4 per culture vessel) were cultured into Majenta Sigma culture vessel (120 ml capacity) containing 25 ml MS medium supplemented with 3% sucrose and solidified with 0.8% agar (w/v). The medium was supplemented with different concentrations and combinations of thidiazuron (TDZ) at 0, 0.5, 1 and 2 mg L^{-1} and indole butyric acid (IBA) at 0, 1 and 2 mg L^{-1}. 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). All the culture bottles were maintained at 25 ± 2°C under a 16 h photoperiod provided by cool white fluorescent light at 40 µmol m^{-2} s^{-1} PPF. The shoot multiplication and growth parameters in terms of number of axillary shoots developed per explant, shoot length of microshoots as well as fresh weight (FW) and dry weight (DW) of the whole explants were recorded after 8 weeks of culture. All measurements were obtained from 12 randomly chosen plantlets. DW was determined after drying for 48 h at 70°C.

Two-to-three centimeter long shoots were used for rooting in half strengths of both MS basal medium and woody plant medium (WPM) (Lloyd and McCown, 1980) supplemented with different concentrations of IBA at 0, 1, 3 and 5 mg L^{-1}. IBA was added to the media prior to autoclaving 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). The cultures were kept at 25°C and 40 µmol m^{-2} s^{-1} PPF (16 h/day). Rooting parameters were recorded after 4 weeks in culture from 12 randomly chosen plantlets.

Column chromatography, thin layer chromatography and NMR

Field leaves material and *in vitro* plant material were oven-dried at 60°C for 24 h. Extracts were prepared by grinding 608.16 g DW of field material and 5.58 g DW of *in vitro* plant material into a fine powders using a grinder. All powders were extracted twice in ethyl acetate (Musabayane et al., 2005) and the extracts were filtered through Whatman No. 1 filter paper. Each filtrate was concentrated in a corning vacuum rotary evaporator under reduced pressure at

64ºC to yield the crude extracts.

The crude extract of the field material (400 mg) was subjected to column chromatography using a glass column (40 × 2 cm) filled with Merck silica gel 60 (0.04 to 0.063 mm) and eluted with a gradient of hexane in ethyl acetate (9: 1; 8: 2; 7: 3; 6: 4). 311 fractions (2 ml each) were collected and analyzed for the presence of BA, OA and UA, then regrouped on the basis of analytical TLC into 26 fractions. The crude extract of *in vitro* plant material (190 mg) was prepared for column chromatography as above, except that a smaller column (40 × 1.1 cm) was used. Two hundred and eighteen fractions of 2 ml each were collected and analyzed for the presence of BA, OA and UA and then grouped into 15 fractions based on analytical TLC similarity, referenced Sigma standards. The eluted fractions and standards of BA, OA, and UA (Sigma) were spotted on pre-coated aluminum plates, Silica gel 60 F_{254} (Merck) and run in a solvent comprised of *n*-hexane: Ethyl acetate (7: 3). The presence of the standards was visualized following spraying with a mixture of absolute alcohol, sulphuric acid and *p*-anisaldehyde (90: 5: 5) (Musabayane et al., 2005), as bright purple, green and blue colourations after drying the plates in a laboratory oven at 105°C for 5 min. The developed TLC plates were immediately photographed using a Sony Cybershot digital camera. Combined fractions of the similar compound following TLC analysis were left to dry at room temperature and the dry mass recorded after 3 days. Ten milligram of each dried sample was dissolved in chloroform-D and NMR analyzed using a Bruker Advance III 400 MHz instrument.

Experimental design and data analysis

Experiments were set up in a completely randomized design. Data were subjected to Duncan's multiple range test (Duncan, 1955) and ANOVA using SAS program (Version 6.12, SAS Institute Inc., Cary, USA).

RESULTS AND DISCUSSION

A 100% of *S. cordatum* seeds were germinated within 2 to 3 days of culture on MS medium without PGRs. Following subculture, healthy rooted green plantlets were developed within 2 weeks (Figure 1A and B). Recalcitrant seeds impose serious storage and conservation problems due to their desiccation and chilling sensitivity, and germination of *S. cordatum* in the field is achieved with difficulty. Although detailed information on seed viability in this species is lacking, it is clear that the seeds do not undergo maturation drying during the final phase of seed development and are thus shed in moist condition. Viability, which may last for only one day may be lost following desiccation below a critical level, as recalcitrant seeds are intolerant to both drying and low temperatures (Probert and Brierley, 1989). Plants that produce recalcitrant seeds are typically restricted to aquatic environments or humid tropical areas, as is the coastal region of KwaZulu-Natal where *S. cordatum* occurs. Germplasm conservation through plant tissue culture techniques therefore provides a means of preserving endangered and species that produce recalcitrant seeds that are difficult to store.

Of the various TDZ and IBA combinations tested for shoot multiplication (Table 1), MS medium with 0.5 mg L^{-1} TDZ and 1.0 mg L^{-1} IBA proved to be the most effective treatment (Figure 1 C) in terms of number of shoots per explant (4.3) and shoot length (6.2 cm). TDZ alone at 0.5 mg L^{-1} produced the highest shoot length, FW and DW per explant. MS medium without PGRs had the lowest number of shoots, shoot length, FW and DW per explants. Exogenous application of cytokinins and auxins has been known to increase shoot proliferation in many plant species (George, 1993) and in *Syzigium species* such as *S. alternifolium* (Sha Villa Khan et al., 1997) and *S. cumini* (Yadav et al., 1990; Remashree et al., 2007). TDZ is considered to be one of the most active PGRs for shoot induction (Murthy et al., 1998). TDZ-induced morphogenesis probably depends on the levels of hormones, and also modulates the endogenous auxin levels. The effect depends on the concentration and the duration of its application. TDZ-induced shoot organogenesis from different explants of many recalcitrant species as well as from medicinal plants has been reported earlier (Thomas, 2003; El-Mahrouk et al., 2010; Dewir et al., 2010). However, TDZ is effective for some species (Heuttenan and Preece, 1993) but not effective for others (Dewir et al., 2006; Mitras et al., 2009), that is, it is species specific.

There was a considerable rooting activity in *S. cordatum* which differed significantly amongst the media type and IBA concentration (Table 2). The best rooting medium was ½ MS or ½ WPM supplemented with 1 mg L^{-1} IBA in which 92% of shoots were rooted with an average of 3.7 roots per plantlet (Figure 1D). Clearly, the rooting percentage was decreased when media were supplemented with ≥ 1 mg L^{-1} IBA. However, the highest number of roots per plantlet (5.2), FW per plantlet (0.274 g) and DW per plantlet (0.041 g) were recorded in ½ MS supplemented with 3 mg L^{-1} IBA. The highest root length (3 cm) and shoot length (4.1 cm) were recorded in ½ MS without IBA. Inducing of roots has previously been a limiting factor in the micropropagation of woody species (Nemeth, 1986). *In vitro* shoots of *S. alternfolium* failed to form roots on ½ MS basal medium (Sha Valli Khan et al., 1997). IBA was shown to significantly affect rooting in *S. alternifolium* (Sha Valli Khan et al., 1997) and *S. cumini* (Remashree et al., 2007) in which a maximum rooting percentage (70%) was obtained at 1.0 mg l^{-1} for both species. IBA has successfully been used in root induction for other species (Mereti et al., 2002; He et al., 2007; Dewir et al., 2010). From the aforementioned results, it is clear that an exogenous supplement of auxins is necessary to improve rooting in *S. cordatum* since

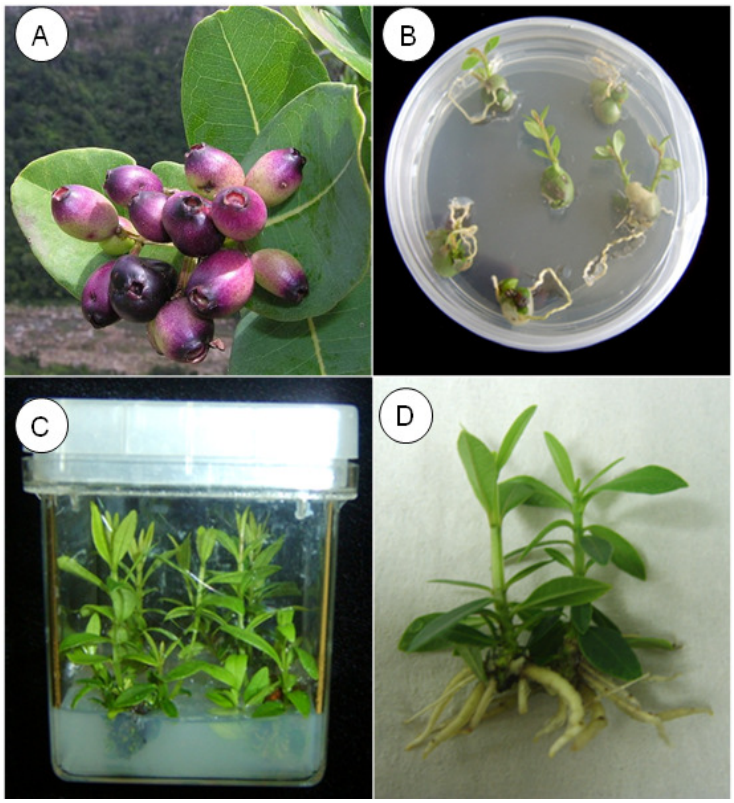

Figure 1. *in vitro* culture of *S. cordatum*. (A) Intact seeds used as plant material. (B) seed germination *in vitro*. (C) Shoot multiplication. (D) *In vitro* rooting.

Table 1. Effect of TDZ and IBA on shoot multiplication and growth of *S. cordatum* using nodal explants after 8 weeks in culture.

PGRs (mg l^{-1})		No. of shoots	Shoot length	Fresh weight	Dry weight
TDZ	IBA	/explant	(cm)	/ explants (mg)	/explants (mg)
MS without PGR_s		1.2 d[Z]	2.7 f	0.091 d	0.018 d
0.5	0.0	2.6 bc	6.1 a	1.199 a	0.146 a
	1.0	4.3 a	6.0 a	0.909 abc	0.114 abc
	2.0	2.9 bc	4.4 cd	0.560 c	0.064 cd
1.0	0.0	2.7 bc	4.7 bcd	0.792 bc	0.108 abc
	1.0	3.3 ab	5.7 ab	0.954 abc	0.127 ab
	2.0	3.6 ab	5.1 abc	0.794 bc	0.109 abc
2.0	0.0	3.4 ab	4.1 cde	1.036 ab	0.141 ab
	1.0	2.8 bc	4.0 de	0.995 ab	0.126 ab
	2.0	2.0 cd	3.3 ef	0.788 bc	0.083 bc
Significance[y]					
TDZ Conc. (A)		NS	***	NS	NS
IBA Conc. (B)		NS	**	*	**
A × B		**	NS	NS	NS

[Z] Mean separation within column by Duncan's multiple range test at 5% level; [y] NS, *, **, *** not significant or significant at $P \leq 0.05$, 0.01 and 0.001, respectively.

Table 2. Effect of media salt strength and IBA concentrations on rooting of *S. cordatum* after 4 weeks in culture.

Treatment		Rooting	No. of roots	Root	Shoot	Fresh weight	Dry weight
Media	**IBA(mg L^{-1})**	**(%)**	**/plantlet**	**length (cm)**	**length (cm)**	**/ explants (g)**	**/explants (g)**
1/2 MS	0	67 ab[z]	2.6 cd	3.0 a	4.1 a	0.146 bc	0.030 ab
	1	92 a	3.7 abc	1.6 b	3.1 bc	0.216 ab	0.026 bc
	3	83 ab	5.2 a	1.2 bc	3.4 b	0.274 a	0.041 a
	5	25 c	4.0 ab	0.5 c	2.2 d	0.086 c	0.016 c
1/2 WPM	0	58 abc	2.1 d	2.5 a	3.1 bc	0.120 bc	0.027 bc
	1	92 a	3.7 abc	1.4 b	2.7 cd	0.178 bc	0.020 bc
	3	75 ab	3.3 bcd	1.4 b	2.3 d	0.150 bc	0.027 bc
	5	50 bc	4.3 ab	0.5 c	2.4 d	0.102 c	0.023 bc
Significance[y]							
Media type (A)		NS	NS	NS	***	*	NS
IBA Conc. (B)		**	*	***	***	***	**
A × B		*	NS	NS	**	NS	NS

[z] Mean separation within column by Duncan's multiple range test at 5% level; [y] NS, *, **, *** not significant or significant at P 0.05, 0.01 and 0.001, respectively.

Table 3. Column chromatography, TLC and NMR analysis of field leaves *and in vitro* plant material of *S. cordatum*

Fractions	TLC plate analysis	NMR analysis and remarks
Field leaves		
1 - 84	1 spot; R_f 0.98	Not identified; yellowish powder
85 - 112	1 spot; R_f 0.76	Not identified; yellow orange powder
113 - 137	1 blue spot; R_f 0.65	Pure BA white powder
138 - 144	2 spots; R_f 0.65 and 0.48	Mixture with traces of BA and OA
145 - 150	1 tailing spot; R_f 0.48	OA (major) and UA
151 - 302	1 green spot; R_f 0.48	OA and UA (major)
303 - 311	2 spots; R_f 0.35 and 0.27	Not identified
***In vitro* plant material**		
1 -36	1 spot; R_f 0.94	Not identified; yellowish powder
36 - 48	1 spot; R_f 0.76	Not identified; greenish powder
49 - 99	2 spots; R_f 0.76 and 0.64	Mixture with traces of BA
100 - 104	1 blue spot; R_f 0.64	Pure BA white powder
105 - 112	1 green spot; R_f 0.48	Mixture of OA and UA
113 - 144	1 green spot; R_f 0.48	UA white powder
145 - 218	2 spots; R_f 0.48 and 0.35	Not identified; cream white powder

endogenous auxin levels are not adequate.

Table 3 summarizes the results obtained on collection of fractions and NMR analysis of the eluents corresponding to reference samples of BA, OA and UA for both field leaves and *in vitro* plant materials. The early fractions (1 to 112) of the field leaves following column separation were chlorophylls and therefore were not further analyzed. The fractions number 113 to 137 contained pure BA. Fractions 138 to 144 contained mixtures of BA and OA, while fractions 145 to 150 contained

mainly OA only. Fractions 151 – 302 corresponded to OA and UA. Fractions 303 – 311 were polar fractions and not further analyzed. The early fractions 1 to 36 of the *in vitro* leaves were chlorophylls and therefore were not further analyzed. Fractions 37 to 48 were steroids. Fractions 49 to 99 did not contain the reference compounds.

Fractions 100 to 104 corresponded to BA. Fractions 105 to 112 were unknowns. Fractions 113 to 144 (ST /41 (xv)) corresponded to OA and UA. Fractions 145 to 218 were mixture of un-analyzed polar compounds. Samples and standards (BA, OA, UA - Sigma), produced identical spectra following H^1 and C^{13} NMR analysis. The aforementioned confirms the presence of the vitally important BA and OA in *S. cordatum*, a medicinal plant used widely in South Africa and further supports the early report of the existence of pentacyclic triterpenoids (Candy et al., 1968).

The present study is the first reported on *in vitro* propagation of *S. cordatum*. Through tissue culture, the cryopreservation of shoot tips and meristems present distinct conservation opportunities. The presence of betulinic acid in the *in vitro* plant material can be exploited for the mass production of this compound using bioreactor technology.

ACKNOWLEDGEMENTS

Research funds were made available by the University of KwaZulu-Natal and the National Research Foundation, South Africa.

REFERENCES

Candy HA, McGarry EJ, Pegel KH (1968). Constituents of *Syzygium cordatum*. Phytochem., 7: 889-490.

Cheruiyot C, Kioko JI, Berjak P (2004). Effects of temperature and moisture content on the short-term storage of *Syzygium cordatum*. Conference Abstracts. S. Afr. J. Bot., 70: 358.

De Clercq E (2000). Novel compounds in preclinical/early clinical development for the treatment of HIV infections. Rev. Med. Virol., 10: 255–277.

Dewir YH, Chakrabarty D, Hahn EJ, Paek KY (2006). A simple method for mass propagation of *Spathiphyllum cannifolium* using an airlift bioreactor. *In Vitro* Cell. Dev. Plant, 42: 291-197.

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

Duncan DB (1955). Multiple range and multiple F tests. Biom., 11: 1-42

El Mahrouk ME, Dewir YH, Omar AMK **(**2010). *In vitro* propagation of adult strawberry tree (*Arbutus unedo* L.) through adventitious shoots and somatic embryogenesis. Propag. Ornam. Plants, 10: 93-98.

George EF (1993). Plant Propagation by Tissue Culture. Part 1: The Technology. Exegetics Limited, Edigton.

He SS, Liu CZ, Saxena PK (2007). Plant regeneration of an endangered medicinal plant *Hydrastis canadensis* L. Sci. Hortic. Amsterdam, 113: 82–86.

Heuttenan CA, Preece JE (1993). Thidiazuron: A potent cytokinin for woody plant tissue culture. Plant Cell Tiss. Org. Cult., 33: 105-119.

Lloyd G, McCown B (1980). Commercially-feasible micropropagation of mountain laurel, *Kalmia latifolia,* by use of shoot-tip culture. Comb. Proc. Int. Plant Prop. Soc., 30: 421-427.

Ma C, Nakamura N, Miyashiro H, Hattori M, Shimotohno K (1999). Inhibitory effects of constituents from *Cynomorium songaricum* and related triterpene derivatives on HIV-1 protease. Chem. Pharm. Bull. (Tokyo), 47: 141-145.

Mathabe MC, Nikolova RV, Lall N, Nyazemac NZ (2006). Antibacterial activities of medicinal plants used for the treatment of diarrhoea in Limpopo Province, South Afr. J. Ethnopharmacol., 105: 286–293.

Mathew MK, Hariharan M (1990). *In Vitro* multiple shoot regeneration in *Syzygium aromaticum.* Ann. Bot. London, 65: 277-279.

Mereti M, Grigoriadou K, Nanos GD (2002). Micropropagation of the strawberry tree, Arbutus unedo L. Sci. Hortic. Amsterdam, 93: 143–148.

Mitras D, Kitin P, Iliev I, Dancheva D, Scaltsoyiannes A, Tsaktsira M, Nellas C, Rohr R (2009). *In vitro* propagation of *Fraxinus excelsior* L. by epycotyls. J. Biol. Res., 11: 37-48.

Murashige T, Skoog FA (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant., 15: 473–479.

Murthy BNS, Murch SJ, Saxena PK (1998). Thidiazuron: a potential regulator of *in vitro* plant morphogenesis. *In Vitro* Cell. Dev. Plant, 34: 267- 275.

Musabayane CT, Mahlalela N, Shode FO, Ojewole JAO (2005). Effects of *Syzygium cordatum* (Hochst.) [Myrtaceae] leaf extract on plasma glucose and hepatic glycogen in streptozotocin-induced diabetic rats. J. Ethnopharmacol., 97: 485-490.

Nemeth G (1986). Induction of rooting. In: Bajaj, Y.P.S. (Ed.), Biotechnology in Agriculture and Forestry-Trees, Springer-Verlag, NewYork, 1: 49-64.

Probert RJ, Brierley ER (1989). Desiccation Intolerance in Seeds of *Zizania palustris* is Not Related to Developmental Age or the Duration of Post-harvest Storage. Ann. Bot. London, 64: 669-674.

Remashree AB, Varghese TT, Raghu AV, Nabeesa E, Neelakandan N (2007). Micropropagation of *Syzigium Cumini* (Linn) Skeels. A multipurpose tree. Res. J. Bot., 2: 208-213.

Selzer E, Pimentel E, Wacheck V, Schlegel W, Pehamberger H, Jansen B, Kodym R (2000). Effects of betulinic acid alone and in combination with irradiation in human melanoma cells. J. Invest. Dermatol., 114: 935–940.

Sha V, Khan PS, Prakash E, Rao KR (1997). *In vitro* micropropagation of an endemic fruit tree *Syzygium alternifolium* (Wight) walp. Plant Cell Rep., 16: 325-328.

Sha V, Khan PS, Hausman JF, Rao KR (1999). Effect of Agar, MS Medium Strength, Sucrose and Polyamines on *in vitro* Rooting of *Syzygium alternifolium*. Biol. Plant, 42: 333-340.

Soler F, Poujade C, Evers M, Carry JC, Henin Y, Bousseau A, Huet T, Pauwels R, De CE, Mayaux JF, Le PJB, Dereu N (1996). Betulinic acid derivatives: a new class of specific inhibitors of human immunodeficiency virus type 1 entry. J. Med. Chem., 39: 1069–1083.

Thomas TD (2003). Thidiazuron induced multiple shoot induction and plant regeneration from cotyledonay explants of mulberry. Biol. Plant, 46: 529-533.

Van Wyk BE, Van OB, Nigel G (1997). Medicinal Plants of South Africa, Briza Publications, Pretoria.

Van WBE, Nigel G (2000). People's Plants: A guide to Useful Plants of Southern Africa, Briza Publications, Pretoria.

Yadav V, Madan L, Jaiswal VS (1990). *In vitro* micropropagation of the tropical fruit tree *syzygium cuminii* L. Plant Cell Tiss. Org. Cult., 21: 87-92.

Zuco V, Supino R, Righetti SC, Cleris L, Marchesi E, Gambacorti PC, Formelli F (2002). Selective cytotoxicity of betulinic acid on tumor cell lines, but not on normal cells. Cancer Lett., 175: 17-25.